# Studies on transamination in microorganisms( Dissertation_全文 )

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# STUDIES
# ON
# TRANSAMINATION IN MICROORGANISMS

BY

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## List of Abbreviations

| | | | |
|---|---|---|---|
| α-ABA | α-Aminobutyric acid | Phe | Phenylalanine |
| γ-ABA | γ-Aminobutyric acid | Thr | Threonine |
| α-AisoBA | α-Aminoisobutyric acid | Pro | Proline |
| α-Ala | α-Alanine | Hpro | Hydroxyproline |
| β-Ala | β-Alanine | Ser | Serine |
| Arg | Arginine | Try | Tryptophan |
| Asp | Aspartic acid | Tyr | Tyrosine |
| Asp($NH_2$) | Asparagine | Val | Valine |
| Glu | Glutamic acid | PALP | Pyridoxal-5-phosphate |
| Glu($NH_2$) | Glutamine | KGA | α-Ketoglutaric acid |
| Gly | Glycine | PyA | Pyruvic acid |
| His | Histidine | PPA | Phenylpyruvic acid |
| Leu | Leucine | OAA | Oxaloacetic acid |
| Ileu | Isoleucine | Cys | Cystine |
| Lys | Lysine | | |
| Met | Methionine | | |
| Nval | Norvaline | | |
| Orn | Ornithine | | |

Amino acid, unless otherwise stated, means L-isomer.

## I. Introduction

Neubauer (1) and Knoop (2) about fifty years ago found the conversion of an amino acid to the analogous α-keto acid and the reverse reaction in several animal species. The first definite evidence in animal tissues of enzymes that catalyze such reactions was provided by Braunstein and Kritzmann (3) in 1937, who reported that in minced preparations of pigeon breast muscle, any α-amino acid, with the exception of Gly, could yield its amino group either to KGA to produce Glu or to OAA to produce Asp.

Transamination is now defined as an interconversion between amino acids and keto acids which is taken place by means of an intermolecular transfer of the α-amino group from an amino acid to an α-keto acid, without the intermediate participation of ammonia.

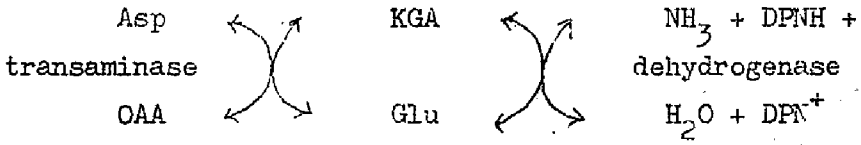
$$H_2N\text{-}\underset{H}{\overset{R_1}{C}}\text{-}COOH + O{=}\overset{R_2}{C}\text{-}COOH \rightleftharpoons O{=}\overset{R_1}{C}\text{-}COOH + H_2N\text{-}\underset{H}{\overset{R_2}{C}}\text{-}COOH$$

It was originally reported that KGA and OAA were capable of accepting the α-amino groups of a large number of amino acids. But, subsequent work by Cohen (4) provided evidence for the existence of only two transaminating systems in swine heart muscle;

(1) Glu + OAA $\rightleftharpoons$ KGA + Asp

(2) Glu + PyA $\rightleftharpoons$ KGA + Ala

These two transamination reactions were most known to exist in animal tissues, in higher plants and in microorganisms. The enzymes (transaminases) that catalyze reaction 1 and 2 have been studied intensively, first those of mammalian muscle and later those in Streptococcus faecalis. Green et al. (5) prepared from swine heart two partially purified enzymes: one catalyzes reaction 1 and may be termed Glu — OAA transaminase: the other catalyzes reaction 2 and is termed Glu — PyA transaminase. The existence of the Glu — OAA transaminase helps to explain the deamination of Asp by tissues that are devoid of aspartase but involve Glu dehydrogenase.




It had been believed on the basis of Cohen's work (4), that enzymatic transamination was mainly limited to reactions between Ala, Glu, Asp and their α-keto analogues. However, from the results of

studies carried out in the last decade, in which more sensitive methods were used, the concept that transamination reaction was limited to only a few amino acids, left unexpected a large number of observations relating to the amination of α-keto acids.

It is known that the labeled $N^{15}$ in ammonia is rapidly incorporated into many amino acids of plant protein (6), and certain α-keto acids are capable of replacing the analogous essential amino acids in supporting the growth of rats (7, 8, 9). Studies carried out in these several years have to led the conclusion that a large number of L-amino acids participate in transamination reactions and that these reactions have an important role in amino acid metabolism. This concept is consistent with certain earlier studies on transamination, as described above, with observations on the rapid incorporation of administered amino acid nitrogen into almost all of the amino acids of animals, and with the ability of animals and microorganisms to utilize the α-keto analogues of some amino acids for growth.

General considerations of the recent progress of studies on transamination and its role in metabolism have been reviewed by Meister (10, 11, 12).

Feldman and Gunsalus (13) showed that several L-amino acids participated in transamination with KGA in such strains of bacteria as Escherichia coli, Pseudomonas fluorescens and Bacillus subtilis and suggested that the feature of transamination reaction in a strain was different from that of any other strain. The occurrence of transaminase activity in Streptococcus faecalis was demonstrated by Snell et al. (14, 15) and studies on the purified Glu — OAA transaminase of the extract from this bacterial strain showed that it was consisted of an enzyme protein and the cofactor PALP, a member of the group of substances designated vitamin $B_6$ (16, 17).

Meister and Tice (18) have reported transamination reaction catalyzed by enzyme preparations of liver and Neurospora crassa between Glu($NH_2$) and a number of α-keto acids, resulting in the formation of KGA, ammonia and the corresponding amino acids. Recent evidence (19, 20) suggests that the transamination occurs prior to deamination, and the observation that the enzyme preparation catalyzes the hydrolysis of α-ketoglutaramate to ammonia and KGA is compatible with the concept that α-keto acid-ω-amide is formed by

transamination, and subsequently split by a specific amidase. Other liver preparations catalyze analogous transamination reaction between Asp($NH_2$) and α-keto acid (21).

Fincham reported that an ω-amino group of Orn was transaminated to KGA to form Glu and glutamic semialdehyde or its cyclic compound, Δ'-pyrroline-5-carboxylic acid, by the extract of Neurospora crassa (22). Roberts et al. (23) found that ω-amino acids such as β-Ala and Y-ABA were also able to participate in transamination reaction with KGA in the presence of the preparation from E. coli.

As it became apparent that there are not only the typical reaction between KGA, PyA or OAA and L-amino acids, but also transamination involving ω-amino acids, D-amino acids and amino acid amides, attention has been directed to the kind and specificity of transaminase. However, the fractionation or purification of transaminases has not been carried out along with the discovery of new transamination reactions. In fact, the purification of some transaminases has been succeeded in spite of difficulties in the last several years (24, 25, 26, 27), but all of these studies dealed with the single transaminase system regardless of the relationship and the interaction with the other transaminase systems on the amino acid metabolism of the organisms.

Owing to the occurrence of so many kinds of transaminase reactions in microorganisms, the distribution and specificity of the enzyme reactions have been regarded as the most interesting subject in this region.

While transamination reaction has recently attracted notice from the point of view of the industrial production of more useful amino acids by these transaminases from keto acids and crude solution containing various kinds of amino acids. Studies on the formation of Glu in high yield from some amino acids and KGA produced by means of the oxidative fermentation with the bacteria of coli-aerogenes type, were carried out by Katagiri and Tochikura (28).

In the present paper, the new method of determination of amino acids to be suitable for the assay of transaminase activity, by means of circular paper chromatography and ninhydrin reaction involving chelating with copper ion, and studies on the distribution of various kinds of transaminases in bacteria, fungi, fungi imperfecti and yeasts, were presented and the general survey and discussion

on the characteristic distribution in each organism was made from the standpoint of the comparative biochemistry. Studies on the partial purification and fractionation of the enzymes and their properties were also described. It is noteworthy that, among various transamination observed in these microorganisms, the occurrence of two new transaminase systems were found in bacteria; one catalyzes the reaction between KGA and taurine, the other catalyzes the transamination between KGA and Lys. The Lys — KGA transaminase, which is most active among transaminase systems demonstrated in Flavobacterium fuscum, was partially purified, and the properties of the enzyme and the keto analogue of Lys transaminated were studied.

II. Determination of Amino Acids

The establishment of the convinient method of determination of amino acid was required to assay the activity of transaminase. The use of paper chromatography as a quick, convinient, and accurate means of qualitative analysis immediately raised the question of the possibility of quantitative paper chromatography and since the earliest work by Naftalin (29), enormous studies on the quantitative determination of amino acid by the aid of this technique have been demonstrated. Nevertheless, almost all of these methods recommended were too complicated to apply to the investigation of transaminase reactions in a large number of microorganisms and did not so satisfy the requirement of accuracy.

Circular paper chromatography described originally by Rutter (30), was widely developed by Giri and his collaborators (31, 32, 33, 34). Circular paper chromatography has the advantage of the simplicity of technique and the sharp separation of amino acids.

The most proposals for the quantitative analysis of amino acid are based on the reaction of ninhydrin with amino acid in vitro under a little drastic condition, e.g. on boiling-water bath, after the elution of the chromatograms with some solvent. The high blank values of control appear sometimes in the process and give difficulty to estimate the amount of amino acid with accuracy.

However, little blank values were observed in the reaction system made directly on paper as described below and the intensity of Ruhemann's purple in the eluate was increased by the addition of copper ion.

Experimental

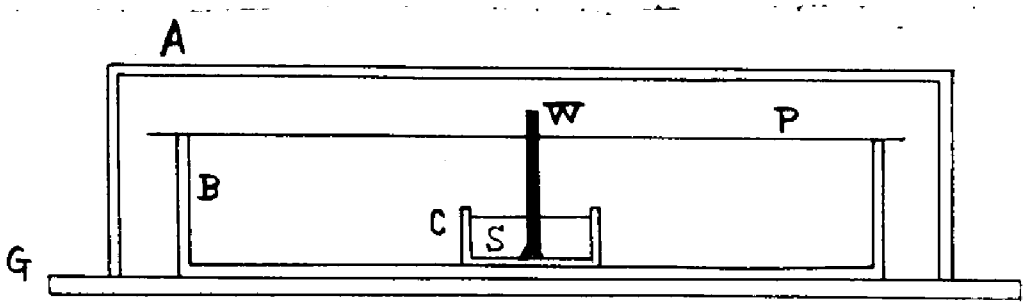
Circular paper chromatography apparatus:

The chromatographic chamber consists of a large Petri dish (28 cm diameter) inverted over a glass plate. The second dish (22 cm diameter) serves as the support for the filter paper disk. The solvent is kept in a small Petri dish (9 cm diameter) set in the center of the chamber. A sketch of the apparatus is shown in Fig. II-1.

A couple of dishes consisted of a larger one (12 cm diameter) and a small one (9 cm diameter) were employed as a apparatus for the

Fig. II-1. Apparatus of circular paper chromatography

A, B, C : Petri dish
P : Filter paper
W : Wick
S : Solvent
G : Glass plate




purpose of chromatographing a small sized filter paper disk.

Filter paper:

Toyo No. 51 was used throughout the experiments. A sheet of paper disk with diameter greater than 22 cm, was employed for separation of amino acids which Rf values were not so different from each other. In the case of chromatographing amino acids that were easily separated, e.g. Glu and Leu with solvent (A), a small sized sheet of circular paper (10 cm diameter) was used and it took only about 30 min for any solvent to run to the end of paper disk in a small apparatus.

Solvents:

The following solvent systems were alternatively employed.

(A) n-Butanol-acetic acid-water (4:1:1)

(B) Ethanol-ammonia-water (18:1:1)

(C) Phenol saturated with water-ammonia (200:1)

Procedure and Results

Aliquots (5-20 µml) of the sample solution containing 0.02-0.15 µmoles of each amino acid were applied from a micro-pipette to six equidistant spots on a pencil-drawn circle with a radius of about 1.5 cm from the center of the paper disk.

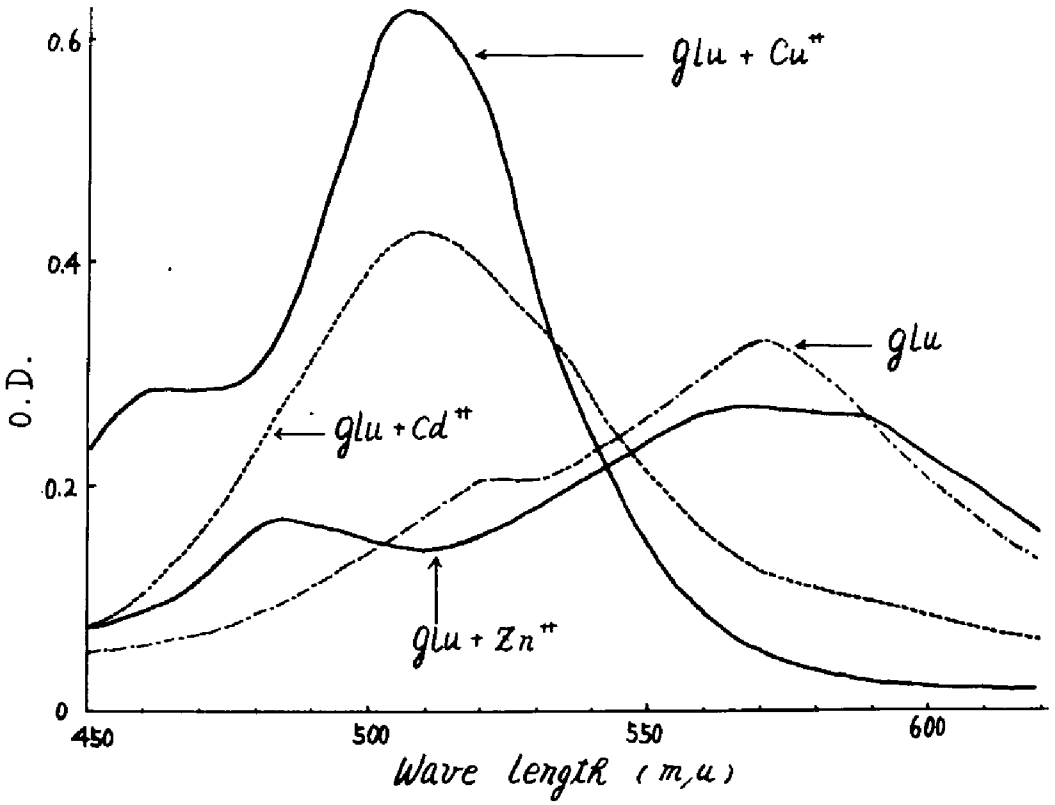
The wick was made from a strip of filter paper (1.5× 4.0 cm), rolled into a rod, cut at the end into a brush and inserted through a pinhole in the center of the paper disk to irrigate it with the solvent.

After the solvent had run, the paper was air-dried. When the solvent (C) was employed, the paper was washed enough with acetone

and dried to remove remaining phenol, because phenol prevents coloration with ninhydrin from good development.

The dried paper was sprayed with 0.5 % ninhydrin solution in 75 % ethanol and heated at 50 °C for 30 min. The colored zones were cut into test tubes and extracted with 5.0 ml of 75 % ethanol containing 0.005 % copper sulfate. The color intensity of the extract was

Fig. II-3 Effect of metal ions on Ruhemann's purple (glutamic acid).




photometrically measured at 500 mμ. Fig. II-2 shows the relationship between amount of amino acid and the optical density under the condition employed in this experiment.

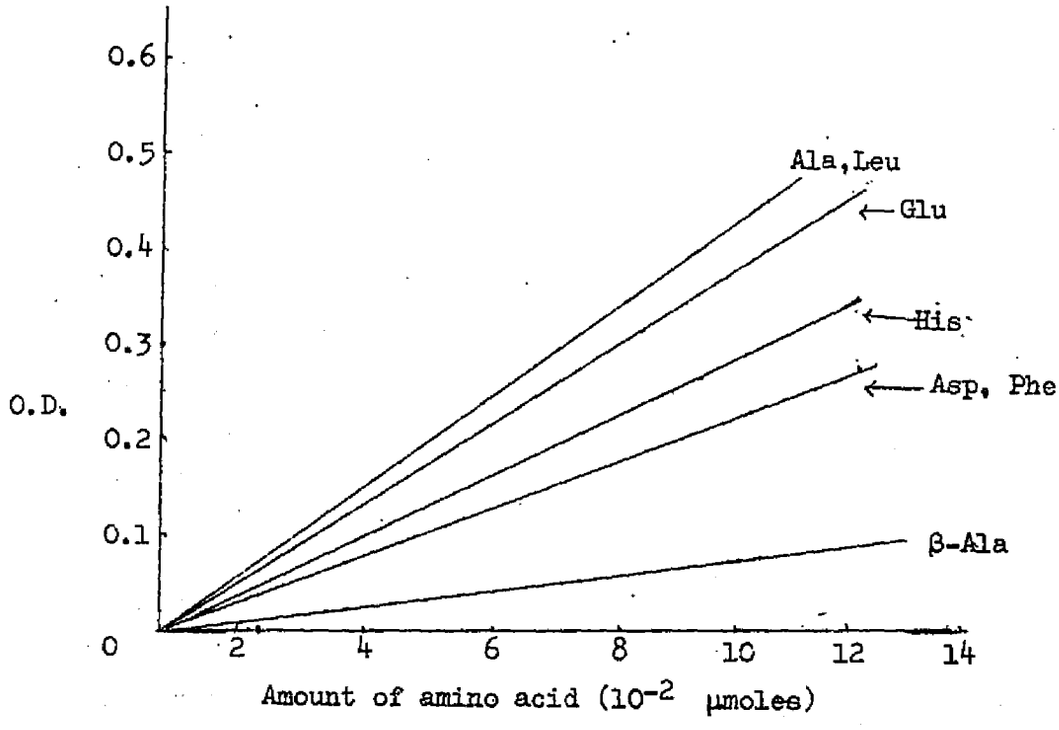
Owing to the addition of copper ion to the amino acid-ninhydrin complex compound, "Ruhemann's purple", in solution, the color was changed from the original violet having a peak at 570 mμ, to the brick red possessing a maximum of its adsorption spectra at 500 mμ and the color intensity increased by 40~50 % (Fig. II-3).

Cadomium ion gave a similar effect at less degree, while the other divalent cations or monovalent cations could not increase the

color intensity.

Each amino acid could be determined with error ± 5 % in such procedure.

Fig. II-2 Relationship between amount of amino acid and optical density.



## III. The Distribution of Transaminase in Bacteria

Since the classical work by Braunstein and Kritsmann (3), a number of studies on transamination were reported on bacteria as well as on animals and plants. Feldman and Gunsalus (13) showed that several L-amino acids participated in transamination with KGA in E. coli, Pseudomonas fluorescens and Bacillus subtilis, and suggested that the distribution of transaminases varied with the kinds of bacteria. The occurrence of transaminases was demonstrated in Streptococcus faecalis by Snell (15) and in Lactobacillus arabinosus by Meister (20). However, the most of these studies dealed with the limited strains and the survey of various transamination in many kinds of bacteria has not yet been made.

In this chapter, the studies on transamination between several α-keto acids and various amino acids catalyzed by the resting cells of many strains of bacteria were reported and the comparative discussion of the characteristic of the distribution of transamination was presented (35, 36).

### Materials and Methods

Microbiological materials:

The bacteria employed in this chapter, unless otherwise stated, were cultivated in a medium prepared as follows; 1.5 % peptone, 1.0 % glucose, 0.4 % $K_2HPO_4$, 0.2 % $KH_2PO_4$, 0.3 % NaCl and 0.02 % $MgSO_4 \cdot 7\ H_2O$ in tap water. The final pH was about 7.2. One hundred ml of the medium was taken in a 500 ml flask and the culture was incubated at 30 °C on a shaker for 24 hrs. The cells harvested by centrifugation were washed twice with 0.85 % sodium chloride solution and suspended in 0.1 M phosphate buffer of pH 7.8. The concentration of the cell suspension was turbidimetrically determined.

Determination of amino acid:

Amino acid was quantitatively determined according to the method described in the previous chapter.

Assay of transaminase activity:

The reaction mixture consisted of 40 μmoles of L-amino acid, 40 μmoles of keto acid, 100 μmoles of phosphate buffer of pH 7.8 and the cell suspension (1~3 mg as dry matter) in a final volume of 2.0 ml. When DL-amino acid was employed as an amino donor, 80 μmoles of the substrate were added to the reaction mixture.

Incubation was carried out at 37°C for 2~4 hr. The reaction was arrested by the addition of ethanol (1 volume), and the cells were centrifuged down and some aliquots of the resulting supernatant were placed on paper disk for analysis of amino acid. The amount of amino acid formed and remaining amino donor was determined as above mentioned. Appropriate blanks and controls which included sets for endogenous reaction were always run. The enzyme activity was expressed as 10x μmoles of amino acid formed by transamination per mg of cells as dry matter per hr.

## Results and Discussion

Transamination between KGA and L-amino acid:

The formation of Glu from KGA and various amino donors by the resting cells of bacteria is listed in Tables III-1, III-2, and III-3.

Table III-1. Transaminase activities between KGA and amino acid by resting cells

| Organism / Amino donor | Flavobac... | Flavobac-terium flavescens | Achromobac-ter superficialis | Achromo-bacter polymorph | Achromo-bacter liquidum |
|---|---|---|---|---|---|
| DL-α-ABA* | 0 | 0 | 0 | 0 | 0 |
| α-AisoBA | 0 | 0 | 0 | 0 | 0 |
| Y-ABA | 0.4 | 0 | 3.3 | 3.6 | 0 |
| L-α-Ala | 0 | 0 | 0 | 0 | 0 |
| β-Ala | 0 | 0 | 1.8 | 1.6 | 0 |
| L-Arg | 1.2 | 0 | 0 | 0 | 1.2 |
| L-Asp | 2.1 | 3.3 | 3.5 | 2.0 | 1.6 |
| L-His | 0 | 0 | 0 | 0 | 0 |
| L-Leu | 4.2 | 4.3 | 0.5 | trace | 3.1 |
| DL-Ileu* | 4.3 | 4.3 | 0.4 | trace | 3.5 |
| L-Lys | 5.7 | 2.3 | 0 | 0 | 4.2 |
| L-Met | 3.1 | 2.0 | 0 | 0 | 3.0 |
| DL-Nval* | 3.9 | 2.7 | 0.5 | 0 | 1.5 |
| DL-Orn* | 2.7 | 1.7 | 1.8 | 1.8 | 1.7 |
| L-Phe | 5.4 | 17.3 | 0.8 | 0.4 | 3.0 |
| L-Try | trace | trace | 0.7 | 0 | 1.7 |
| DL-Val* | 4.0 | 3.3 | 0.4 | 0 | 1.0 |

*: When DL-amino acid was employed as an amino donor, 80 μmoles of the substrate were added to reaction mixture.

Incubation was carried out at 37°C for 4 hr in these experiments. Table III-1 shows that Leu, Ileu, Val, Nval, Lys, Phe, Met and Asp were transaminated with KGA as a very active amino donor in the resting cells of Flavobacterium fuscum, Fl. flavescens and Achromobacter liquidum. Above all, it is interesting that Lys — KGA trans-

amination reaction was most actively presented in the cells of Fl. fuscum and Ach. liquidum, and further observation on this transamination and the enzyme will be described in full detail in the following chapter.

On the contrary, Y-ABA, β-Ala, Orn and Asp were used as an active amino donor to KGA and the other amino acids such as Leu, Phe, Met or Lys were little or not at all transaminated with KGA by the resting cells of Ach. superficialis and Ach. polymorph. β-Ala, Y-ABA and Orn have each an amino group at $\omega$-position and the former two amino acids are known as the decarboxylated product of Asp and Glu, respectively. The fractionation and the properties of these enzymes will be demonstrated bellow. Achromobacter liquidum was rather closer to Fl. fuscum and Fl. flavescens of the different genus than the other strains of its own genous from the view-point of the characteristic of transamination reactions.

Table III-2. Transaminase activities between KGA and amino acid by resting cells

| Amino donor \ Organism | Escherichia coli 2b | Alcaligenes faecalis | Agrobacterium tumefaciens | Xanthomonas citri | Corynebacterium sepedonicum |
|---|---|---|---|---|---|
| DL-α-ABA | 0.4 | 0 | 0 | 1.5 | 1.1 |
| α-AisoBA | 0 | 0 | 0 | 0 | 0 |
| Y-ABA | trace | trace | 0 | trace | 1.0 |
| L-α-Ala | trace | 0 | 0 | 0 | 1.0 |
| β-Ala | 0 | 0 | 0 | 0 | 0 |
| L-Arg | 0 | 0 | trace | 1.6 | 0 |
| L-Asp | 4.6 | 2.1 | 0.7 | 5.1 | 2.1 |
| L-His | 0 | 0 | 0 | 0 | 0 |
| L-Leu | 2.3 | 2.0 | 0.5 | 6.6 | 1.1 |
| DL-Ileu | 2.2 | 1.5 | 0.5 | 5.6 | 1.0 |
| L-Lys | 0 | 0 | 0 | 1.6 | 0 |
| L-Met | 0.9 | 0.8 | 0.5 | 2.6 | 0 |
| DL-Nval | 2.4 | 2.6 | 0.5 | 5.2 | 0.9 |
| DL-Orn | - | - | 0 | 3.3 | 3.5 |
| L-Phe | 3.0 | 2.8 | 0.3 | 10.0 | 0.5 |
| L-Try | 0.7 | 0.8 | 0 | 4.5 | 0 |
| DL-Val | 2.3 | 2.5 | 0.4 | 5.6 | 0.8 |

Gly, Cys, Ser, Thr, Pro, Hpro and ammonium chloride were not capable of participating in transamination with KGA in the resting cells of these bacteria.

Table III-2 indicates that transamination between KGA, and Asp Leu, Ileu, Nval, Val and Phe were actively catalyzed by the cells of E. coli and Alcaligenes faecalis as well as by those of Flavobacterium

but the formers were able to catalyze more actively Asp — KGA transamination than the latters. Lys, which was a highly active amino donor in Flavobacterium fuscum, Fl. flavescens and Ach. liquidum, was not transaminated at all by the cells of these strains.

The transamination systems occurred in Xanthomonas citri resembles generally to those in Flavobacterium fuscum and Fl. flavescens, with the exception of the absence of Lys — KGA transaminase activity and the presence of the active Try — KGA transamination and α-ABA-KGA transamination. It was found that the resting cells of Agrobacterium tumefaciens had little activity in transamination with KGA.

Table III-3. Transaminase activities between KGA and amino acid by resting cells

| Organism / Amino donor | Proteus vulgaris | Pseudomonas fluorescens | Bacillus natto SN | Bacillus roseus | Bacillus thiamino-lyticus |
|---|---|---|---|---|---|
| DL-α-ABA | 1.9 | 1.9 | 7.0 | 5.8 | 4.2 |
| α-AiscBA | 0 | 0 | 0 | 0 | 0 |
| Y-ABA | 0 | 6.0 | 2.4 | 0 | trace |
| L-α-Ala | 3.1 | 3.3 | 8.1 | 5.5 | 2.1 |
| β-Ala | 0 | trace | trace | 0 | 0 |
| L-Arg | 0 | 0.7 | 2.7 | 1.3 | trace |
| L-Asp | 5.3 | 12.5 | 6.8 | 4.5 | 3.3 |
| L-His | 0.6 | 0 | 0 | 0 | 0 |
| L-Leu | 1.1 | 8.0 | 0.8 | 0 | 10.0 |
| DL-Ileu | 1.0 | 3.6 | 0.8 | 0 | 7.0 |
| L-Lys | 0 | 1.6 | trace | 0 | 0 |
| L-Met | 2.0 | 3.3 | 1.0 | 0.8 | 5.0 |
| DL-Nval | 1.7 | 2.6 | 1.5 | 3.5 | 6.9 |
| DL-Orn | 0 | 1.5 | 3.0 | 5.0 | 0.8 |
| L-Phe | 6.8 | 4.0 | 1.0 | trace | 2.0 |
| L-Try | 4.1 | 4.7 | 1.2 | 1.0 | 0.8 |
| DL-Val | 0.8 | 3.2 | 1.3 | 1.2 | 8.5 |

In the resting cells of Corynebacterium sepedonicum shown in Table III-2, and the strains presented in Table III-3, it is noteworthy that α-ABA, α-Ala, Met and Try, which were little or not at all used as amino donors in the other bacteria, were actively transaminated with KGA as well as Asp, while Leu, Ileu, Nval or Val — KGA transamination was faintly observed in these strains except in Pseudomonas fluorescens and Bacillus thiaminolyticus Matsukawa. Glu was highly formed from KGA and α-ABA or α-Ala by the cells of 3 strains of Bacilli. The distribution of transamination in B. natto SN was close to that in B. roseus, e.g. there were active α-ABA — or α-Ala— KGA transaminase system and faint or no Leu, Ileu, Nval or

Val — KGA transamination system.

It is noteworthy that both Asp — KGA and Phe — KGA transamination reactions occur generally in any bacteria investigated in this chapter. This fact suggests that these two reactions have very important role in amino acid metabolism. Leu —, Ileu —, Nval — or Val — KGA transamination was found always in company with each other. These reactions are suggested to be catalyzed by same or similar transaminase. Meister et al. (37) obtained fractions which catalyzed transaminations between the amino acids (or keto analogues) of the following groups; (A) Glu, Asp, Try, Phe and Tyr (B) Glu, Leu, Ileu and Val. The results obtained here on the distribution of transamination reactions in bacteria do not support the existence of transaminase A catalyzing reactions between the amino acids belonging in group (A), but may support the existence of transaminase B.

γ-ABA — KGA, β-Ala — KGA and Lys — KGA transamination reactions were found specifically in the resting cells of some strain. Transamination between PyA, OAA or PPA and amino acid:

The transamination reactions between PyA, OAA or PPA and various amino acids to form Ala, Asp or Phe, respectively, by the resting cells of bacteria were investigated and the results are presented in Tables III-4 and III-5.

Table III-4. Transaminase activities between PyA, PPA and OAA and amino acid by resting cells

| Organism | Agrobacterium tumefaciens | | Proteus vulgaris | | Xanthomonas citri | |
|---|---|---|---|---|---|---|
| donor \ product | α-Ala | Phe | α-Ala | Phe | α-Ala | Asp |
| DL-α-ABA | 0 | 0 | 3.2 | 0 | 0 | 0 |
| γ-ABA | 0 | 0 | 0 | 0 | 0 | 0 |
| L-α-Ala | - | 1.0 | - | 0 | - | 0 |
| β-Ala | 0 | 0 | 0 | 0 | 0 | 0 |
| L-Asp | 0 | 0 | 0.3 | 1.7 | trace | - |
| L-Glu | 0 | trace | 1.0 | 6.4 | trace | 5.8 |
| L-Leu | 0.5 | 2.8 | 0.5 | 0 | 0 | trace |
| L-Lys | 0.3 | 0 | 1.3 | 0 | 0 | 0 |
| DL-Nval | 0.4 | 2.5 | 0.4 | 0 | 1.0 | 0 |
| DL-Orn | 0 | 0 | 1.2 | 0 | 0 | 1.6 |
| L-Phe | 0.4 | - | 0.3 | - | trace | 6.7 |
| L-Try | 0.5 | 0 | 0 | 0 | trace | 1.6 |
| DL-Val | 0.5 | 2.2 | 0.4 | 0 | 0 | trace |

Generally, the activities of these transaminase reactions with

the exception of the reaction systems involving Glu as an amino donor, were lower and the multiplicity of the reactions occurred was poorer than those of the transamination systems containing KGA as an amino acceptor, although the transamination with PPA was more active than that with KGA in the presence of resting cells of Agrobacterium tumefaciens which was demonstrated to have faint activities of transaminase reactions with KGA as indicated in Table III-2.

Table III-5. Transaminase activities between PyA, PPA and OAA and amino acid by resting cells.

| Organism | Pseudomonas fluorescens | | Bacillus natto SN | Bacillus thiaminolyticus | | Bacillus roseus |
|---|---|---|---|---|---|---|
| Amino acid formed | α-Ala | Asp | α-Ala | α-Ala | Asp | α-Ala |
| DL-α-ABA | 3.0 | 0 | 3.0 | 0 | trace | 13.2 |
| γ-ABA | 4.6 | 0 | 0 | 0 | 0 | 0 |
| L-α-Ala | - | 0 | - | - | 0 | - |
| β-Ala | 5.9 | 0 | 0 | 0 | 0 | 0 |
| L-Asp | 0.9 | - | trace | 2.2 | - | trace |
| L-Glu | 3.6 | 1.0 | 0.5 | 2.6 | 6.8 | trace |
| L-Leu | trace | 0 | 0 | 0 | 0 | 0 |
| L-Lys | trace | 0 | 0 | 0 | 0 | 0 |
| DL-Nval | 0 | 0 | 1.0 | 0 | 0 | 0 |
| DL-Orn | 0 | 0 | 0 | 0 | 0 | 0 |
| L-Phe | 0 | 2.5 | 0 | 0 | 0 | 0 |
| L-Try | 0 | 2.8 | 0 | 0 | 2.6 | 0 |
| DL-Val | 0 | 0 | 0 | 0 | 0 | 0 |

In the resting cells of Bacillus natto SN, B. roseus, Pseudomonas fluorescens and Proteus vulgaris, α-ABA — PyA transamination reaction was found to exist actively as well as α-ABA — KGA reaction. Above all, the active transaminations of PyA with β-Ala and γ-ABA having an $\omega$-amino group, besides α-ABA, were shown in the resting cells of Pseudomonas fluorescens. The occurrence of transaminase catalyzing PyA — β-Ala reaction was observed in a strain of Pseudomonas and the properties of the purified enzyme were investigated by Hayaishi et al.(38). PPA — Glu and PPA — Leu, Nleu or Val transamination reactions were demonstrated in Proteus vulgaris and Agrobacterium tumefaciens. Recently, Asai, Aida and Oishi reported studies on the preparation of Phe from PPA and the other amino acids by bacterial transaminase (39).

The results obtained in this experiment show that the distribution and the kind of transamination reactions vary with the species or genus of bacteria and Asp — KGA and Phe — KGA transamination reactions occur generally in all these bacteria tested and may play an important role in the amino acid metabolism, and the transaminations involving KGA or Glu were generally and actively observed as compared with the reactions containing PyA, OAA or PPA as an amino acceptor.


Summary

1. The distribution of transaminases revealing the reactions between α-keto acid such as KGA, PyA, OAA and PPA, and various amino acids was observed with 15 strains of various kinds of bacteria.

2. Asp and Phe were generally found to be active donors of amino group to KGA with all the bacteria employed.

3. Leu, Ileu, Val, Nval and Phe were highly active donors to KGA in the cells of Fl. fuscum, Fl. flavescens, Ach. liquidum, Alcaligenes faecalis, Xanthomonas citri and E. coli 2b.

4. ω-Amino group existing in γ-ABA, β-Ala and Orn revealed powerful transamination with KGA, besides Asp, in Ach. superficialis and Ach. polymorph.

5. Not only Asp, Phe, Orn and Try, but also α-ABA and β-Ala showed higher activity as an amino donor to KGA in the case of Bacillus natto SN, B. roseus, B. thiaminolyticus, Proteus vulgaris, Pseudomonas fluorescens and Corynebacterium sepedonicum.

6. Activity of transamination reaction between L-amino acid and PyA, OAA or PPA was generally lower than that obtained in the case of KGA, excepting high activities of transaminations of Leu, or Nval with PPA in the resting cells of Agrobacterium tumefaciens, of α-ABA with PyA in the cells of Proteus vulgaris and three strains of genus Bacillus and of β-Ala with PyA in the cells of Pseudomonas fluorescens.

IV. Fractionation of Bacterial Transaminase and Their Properties.

In the previous chapter, the distribution and the kind of transamination in various strains and genera of bacteria, were investigated and the results obtained suggest that transamination reactions are characteristic of each organism and their types are able to be classified into some groups.

From the results of these investigations demonstrating the existence of such varieties of the reaction systems of transamination, it would be interesting to point out the kind and the specificity of transaminases.

Extract of E. coli was fractionated by selective adsorption on and then elution from calcium phosphate gel and the three groups of transaminases were separated by Rudman and Meister (37). Two enzymes of these were found to reveal all of the KGA — amino acid transaminase activities of the original extract. Transaminase (A) catalyzed the reaction between Glu and Phe, Tyr, Try or Asp, while transaminase (B) catalyzed transamination between Glu and Leu, Ileu, Nleu, Val or Nval. The amino acids in each group A or B transaminated with others of the same group as well as Glu. The last was a Val and Ala, or α-ABA transaminase.

Recently, Wagner et al. (40) investigated the substrate specificity and some properties of transaminase between PPA and L-amino acid with the extract from Neurospora crassa.

In the present chapter, transaminases in the extract of Fl. fuscum and Ach. superficialis were fractionated and their properties were compared with one another in order to approach the problem of the multiplicity of transaminases. The studies presented in this chapter have been previously reported (35, 36).

## Materials and Methods

Microbiological materials:

Flavobacterium fuscum (Zimmermann, Bergy et al.) and Achromobacter superficialis were employed throughout this work. The method of growing culture and the preparation of washed cells have been mentioned previously in chapter III.

Preparation of the cell-free extract:

The washed cells were suspended in 0.1 M phosphate buffer of pH 7.8 containing 0.01 % 2-mercaptoethanol. The suspension was

treated in a 10 Kc Raytheon sonic oscillator for 15 min., followed by centrifugation at 9,000 r.p.m. for 25 min. The supernatant was employed as the crude extract after dialysis against 0.005 M phosphate buffer of pH 7.8 containing 0.01 % 2-mercaptoethanol, at 4 °C for 15 hr.

Measurement of enzyme activity:

The complete system of the reaction mixture, unless otherwise stated, consisted of 40 μmoles of L-amino acid, 40 μmoles of KGA, 20 μg of PALP, enzyme and 100 μmoles phosphate buffer of pH 7.8 in a total volume of 2.0 ml. Incubation was carried out at 37°C for 30 or 60 min. At the end of the incubation period, 2.0 ml of ethanol was added to stop the reaction. After removal of protein by centrifugation, aliquots of the supernatant were placed on a paper chromatograph for analysis of amino acid. Glu formed and remaining amino donor were estimated as mentioned above. The appropriate controls were always run.

Protein was determined by Lowry's method (41). Activity of the enzyme was expressed as μmoles of amino acid formed per mg of protein per hr.

## Results and Discussion

Fractionation of transaminase of Flavobacterium fuscum:

The cell-free extract of Fl. fuscum was dialyzed against 0.005 M phosphate buffer of pH 7.8 at 4°C for 15 hr. and stored at -5°C for 15 hr. After thawing of the frozen solution, the precipitated residue was removed by centrifugation. The precipitate had no activity of transaminase. The supernatant was brought to 45 % saturation with ammonium sulfate at 4°C after 30 min, the precipitate was removed by centrifugation at 9,000 r.p.m. for 30 min. and then the supernatant was brought to 70 % saturation with ammonium sulfate. The pH of the solution was kept at 7.2~7.8 during the treatment. The precipitate was collected and dissolved in 0.01 M phosphate buffer of pH 7.8. The resulting solution was dialyzed against 0.002 M phosphate buffer of pH 7.8 containing 0.01 % 2-mercaptoethanol at 4°C for 5 hr. After the addition of 1 M acetate buffer of pH 5.3 (0.1 volume) to the solution, the same amount of alumina gel Cγ as enzyme protein was added. The mixture was kept under stirring at 2°C for 10 min. and then the gel was collected by centrifugation. The enzyme was eluted

successively from the gel with 0.1 M phosphate buffers of pH 6.3, 7.0 and 8.0.

On the other hand, the precipitate obtained by 45 % saturation with ammonium sulfate was dissolved in 0.01 M phosphate buffer of pH 7.8 and the solution was dialyzed against 0.005 M phosphate buffer of pH 7.8 at 4°C for 5 hr. The solution was mixed with one tenth volume of 1 M acetate buffer of pH 5.3. The resulting solution was fractionated by selective adsorption on and then elution from alumina gel Cγ as described above.

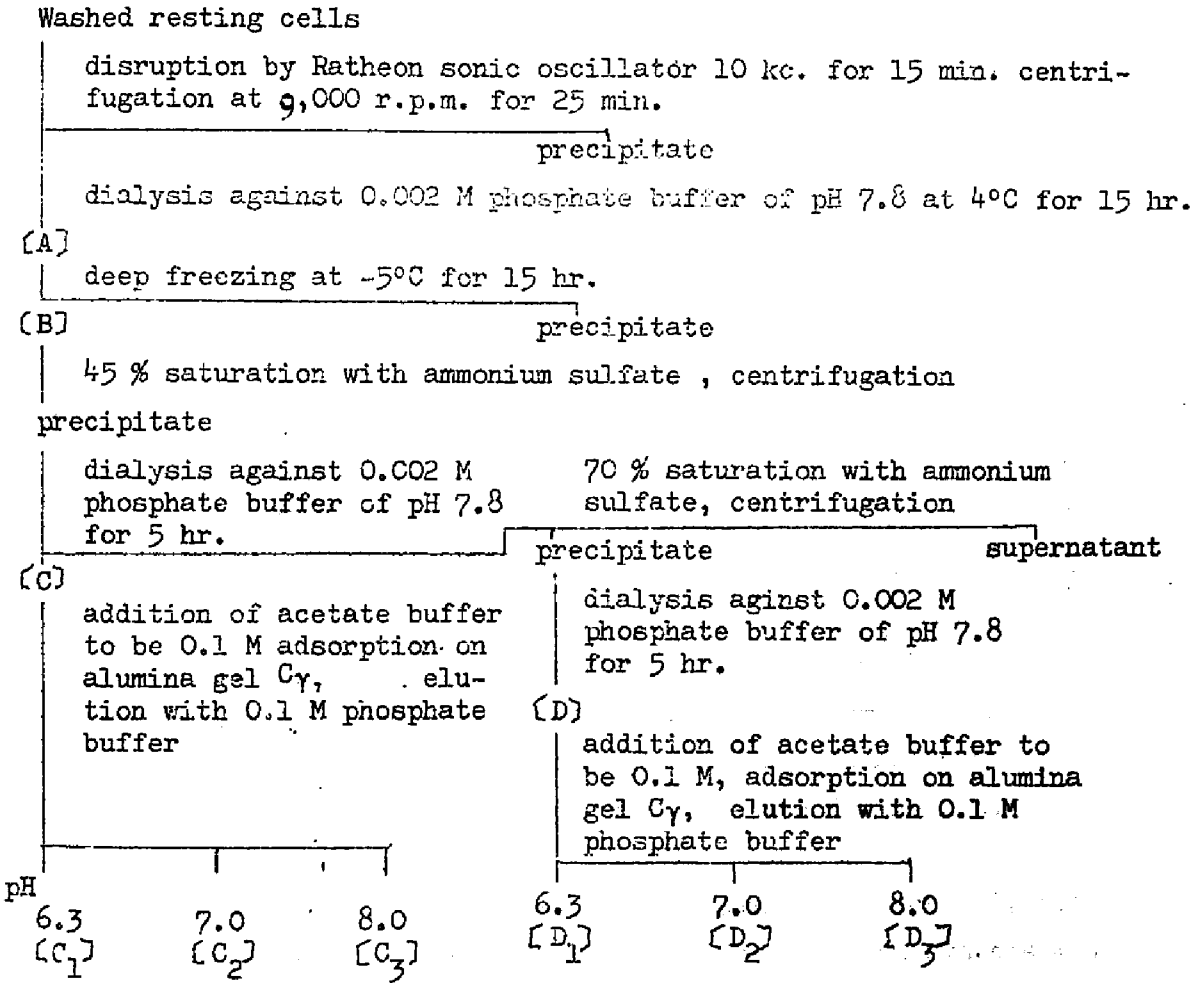
Table IV-1. Activity of transaminase of enzyme fractions of Fl. fuscum

| Fraction / Amino donor | Transaminase activity (Glu, formed(μmoles) per mg of protein per hr) | | | | |
|---|---|---|---|---|---|
| | A | B | C | $C_1$ | $C_2'$ |
| Y-ABA | trace | 0 | 0 | 0 | 0 |
| L-Arg | 0.18 | 0.30 | 0.70 | 0 | 1.10 |
| L-Asp | 0.31 | 0.50 | trace | 0 | 0 |
| L-Leu | 0.61 | 1.48 | 0.92 | 1.21 | 0 |
| DL-Ileu | 0.60 | 1.35 | 1.10 | 1.21 | 0 |
| L-Lys | 0.70 | 2.01 | 5.85 | 14.20 | 14.33 |
| L-Met | 0.20 | 0.40 | 1.13 | 0.50 | 1.81 |
| DL-Nval | 0.40 | 0.91 | 0.62 | 1.34 | 0 |
| L-Phe | 0.81 | 1.97 | 1.50 | 0.62 | 2.21 |
| L-Try | 0.10 | trace | 0 | 1.50 | 0 |
| DL-Val | 0.45 | 0.93 | 0.70 | 0.70 | 0.80 |
| Fraction | $C_3$ | D | $D_1$ | $D_2$ | $D_3$ |
| Y-ABA | 0 | 0 | 0 | 0 | 0 |
| L-Arg | 0.41 | 0 | 0 | 0 | 0 |
| L-Asp | trace | 2.10 | 4.30 | 1.40 | trace |
| L-Leu | 0 | 3.82 | 4.66 | 9.20 | 12.32 |
| DL-Ileu | 0 | 3.80 | 3.90 | 4.86 | 12.10 |
| L-Lys | 6.74 | 6.13 | 8.27 | 13.10 | 12.10 |
| L-Met | 1.10 | 0 | 0 | 0 | 0 |
| DL-Nval | 0 | 1.45 | 3.78 | 5.10 | 4.10 |
| L-Phe | 0 | 6.07 | 8.20 | 8.13 | 6.00 |
| L-Try | 0 | 0 | 0 | 0 | 0 |
| DL-Val | 0 | 1.63 | 3.56 | 4.83 | 3.00 |

Fig. IV-1 and Table IV-1 summerize the steps of the fractionation and the distribution of activity of transaminase of various reaction systems. The activity of the transaminase reaction between KGA and Asp, Phe, Leu, Ileu, Val or Nval, was observed in fraction (D) and the activity of KGA — Met transaminase was found fraction (C). The activity of Lys transaminase appeared in both fractions. The enzymes

observed in fraction (D) were partially separated by selective adsorption on and elution from alumina gel Cγ at different pH. The activities of KGA — Asp and Phe — KGA transaminases were found to be stronger in fraction ($D_1$) than the other fractions, while the activities of Leu, Ileu, Val and Nval transaminases were observed to be powerful in ($D_3$), although the complete fractionation of these enzymes could not be carried out by the procedures mentioned above.

Fig. IV-1. Fractionation proceudre of transaminases of Fl. fuscum




Stability of transaminases against heat treatment:

The crude extract without the addition of 2-mercaptoethanol was heated at 50°C for 30, 60 or 120 min. and then cooled immediately with ice. The activities of various transaminase systems of the heat-treated extracts were determined, and the stability of enzymes against such treatment was compared. As shown in Table IV-2, the activity of transaminase involving Leu, Ileu, Nval, Val or Orn as an amino donor was kept relatively stable against heating and during

long storage at lower temperature, while Asp — KGA and Phe — KGA transaminase activities were easily lost. These facts would due to the sensibility of functional -SH groups of the enzymes or to the different degree of resolution of transaminase to coenzyme and labile apoenzyme.

Effect of inhibitors on transaminase activity of the enzyme preparation of Fl. fuscum :

The effect of isonicotic acid hydrazide (INAH) and hydroxylamine on the activities of Asp —KGA, Leu — KGA and Phe — KGA transaminases were investigated. As demonstrated in Table IV-3,

Table IV-2. Stability of transaminase of Fl. fuscum against heating

| Heating / Amino donor | Relative Activity: No treatment | at 50°C for 30 min | at 50°C for 60 min | at 50°C for 120 min | at -5°C for 17 days |
|---|---|---|---|---|---|
| L-Asp | 100 | 56 | 21 | 0 | 17 |
| L-Leu | 100 | 100 | 47 | 14 | 91 |
| DL-Ileu | 100 | 100 | 48 | 18 | 92 |
| L-Lys | 100 | 92 | 71 | 24 | 78 |
| L-Met | 100 | 88 | 47 | 0 | 65 |
| DL-Nval | 100 | 100 | 44 | 11 | 87 |
| DL-Orn | 100 | 100 | 66 | 20 | 77 |
| L-Phe | 100 | 100 | 28 | 0 | 62 |
| L-Try | 100 | 50 | 0 | 0 | 0 |
| DL-Val | 100 | 90 | 40 | 8 | 90 |

Leu — KGA transaminase was most remarkably inhibited by these two inhibitors, while they gave little inhibition on Phe — KGA enzyme and especially on Asp — KGA transaminase which was found to be most sufficiently affected by heating or by storage for long period.

Effect of pH on enzyme activity:

As illustrated below (in Lys — KGA transaminase reaction), the optimal pH for Leu —, Phe — and Asp — KGA transaminase systems was about 7.8, 8.7 and 7.2 ~ 7.8, respectively. Borate buffer and acetate buffer were employed in place of phosphate when pH was higher than 8.0 and lower than 5.0, respectively.

These results obtained above suggest that the various transamination reaction are not attributed to single enzyme or a few enzymes having wide group specificity, but to several different enzymes, although the complete fractionation of transaminase of the extract of Fl. fuscum was not carried out. Leu —, Ileu —,

Nval — and Val — KGA transaminase activities were observed almost in parallel with each other in the preparation of this strain and these observations coincide the concurrence of these transaminations in the resting cells as mentioned in the previous chapter, and may support Meister's finding and suggestion on transaminase (B) (37).

Table IV-3. Effect of inhibitor on transaminase activity of Fl. fuscum.

| | | Degree of inhibition (%) | | |
|---|---|---|---|---|
| | concentration | KGA — Asp | KGA — Leu | KGA — Phe |
| Isonicotic acid hydrazide | $5\times10^{-4}$M | 0 | 0 | 0 |
| | $5\times10^{-3}$ | 5 | 12 | 0 |
| | $5\times10^{-2}$ | 28 | 52 | 31 |
| Hydroxylamine | $5\times10^{-5}$ | 11 | 32 | 8 |
| | $5\times10^{-4}$ | 56 | 87 | 68 |
| | $5\times10^{-3}$ | 100 | 100 | 100 |

Preincubation of the reaction mixture was carried out before the the addition of substrates, at 37°C for 15 min.

Fractionation of transaminase of Achromobacter superficialis:

The transaminases of the resting cells or the extract of Ach. superficialis were specifically active for the Asp — KGA, β-Ala — KGA, Y-ABA — KGA and Orn — KGA reaction systems, while the activity of the transaminase reaction with the other amino acids was faint. The transaminases of the crude extract were fractionated and partially purified in order to investigate the kind and specificity of the enzyme. The dialyzed extract was kept at 60 °C for 10 min. and then cooled with ice. After removal of the precipitate by centrifugation, the supernatant was mixed with 1.5 % protamine sulfate solution in the proportion of 1 ml to 100 mg protein of enzyme. The precipitate was removed and the resulting supernatant was brought to 25 % saturation with ammonium sulfate. The supernatant obtained by centrifugation was brought to 75 % saturation with ammonium sulfate and the precipitate was collected and dissolved to 0.01 M phosphate buffer of pH 7.8. The solution was dialyzed against 0.002 M phosphate buffer of pH 7.8 containing 2-mercaptoethanol at 4°C for 5 hr. One M acetate buffer of pH 5.3 (0.1 volume) was added to the solution and alumina gel $C_\gamma$ was then added under stirring. The enzymes were eluted successively from the gel with 0.1 M phosphate buffer of pH

6.3, 7.0 and 8.0.

As shown in Fig. IV-2 and Table IV-4, γ-ABA — KGA and Asp — KGA transaminases were purified to about 20 fold and were hardly fractionated by alumina gel $C_\gamma$ treatment and by selective elution. Activities

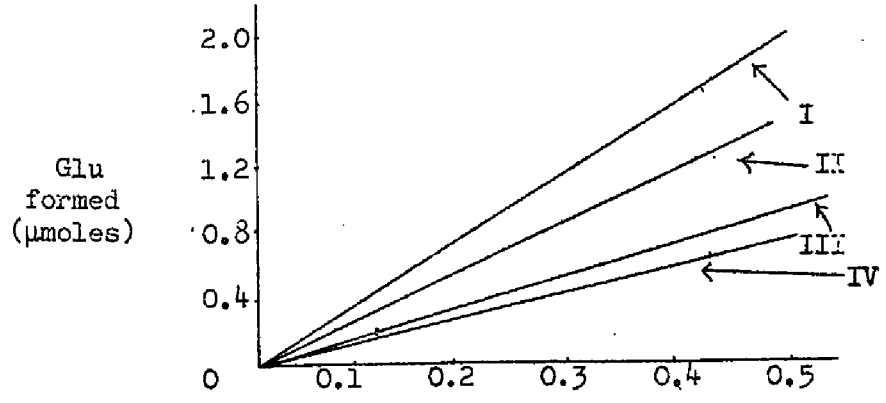
Table IV-4. Transaminase activity of fraction of Achromobacter superficialis

| Reaction system | γ-ABA — KGA | | Asp — KGA | | Orn — KGA | | β-Ala — KGA | |
|---|---|---|---|---|---|---|---|---|
| fraction | Activity | Yield % | Activity | Yield % | Activity | Yield % | Activity | Yield % |
| A | 1.8 | 100 | 1.6 | 100 | 0.9 | 100 | 0.63 | 100 |
| B | 3.9 | 90 | 3.3 | 86 | 2.1 | 88 | 1.88 | 82 |
| C | 13.1 | 48 | 8.4 | 63 | 5.3 | 32 | 4.20 | 36 |
| D | 19.5 | 36 | 20.1 | 43 | | | | |
| $D_1$ | 21.3 | 6 | 31.1 | 8 | | | | |
| $D_2$ | 28.0 | 15 | 26.3 | 18 | | | | |
| $D_3$ | 18.3 | 4 | 21.4 | 5 | | | | |

of Orn — KGA and β-Ala — KGA transaminase were less stable and disappeared during the processes.

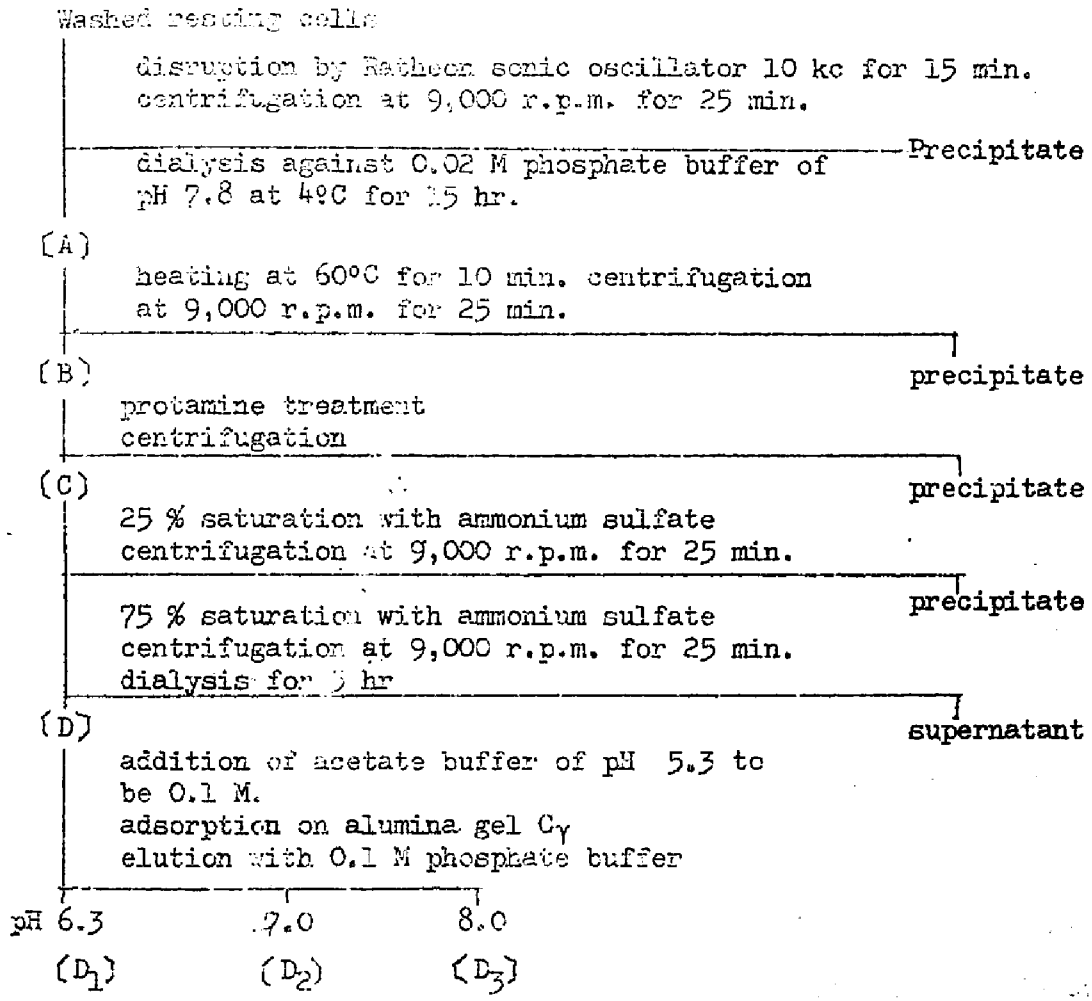
It was found that transaminases catalyzing these four reactions could separate at least into two groups, one involved γ-ABA — KGA and Asp — KGA transamination, while the other involved β-Ala — KGA and Orn — KGA reactions. With the preparations thus obtained, some properties of the enzymes and their characteristic difference were investigated. The formation of Glu was found to be progressed in

Fig. IV-3, Effect of concentration of enzyme protein on the transaminase activity.
I. γ-ABA —KGA transaminase system
II. Asp — KGA
III. Orn — KGA
IV. β-Ala — KGA



parallel with the amount of enzyme protein (Fig. IV-3) and with incubation time under the conditions adopted.

Fig. IV-2. Fractionation procedure of transaminase of Ach. superficialis .

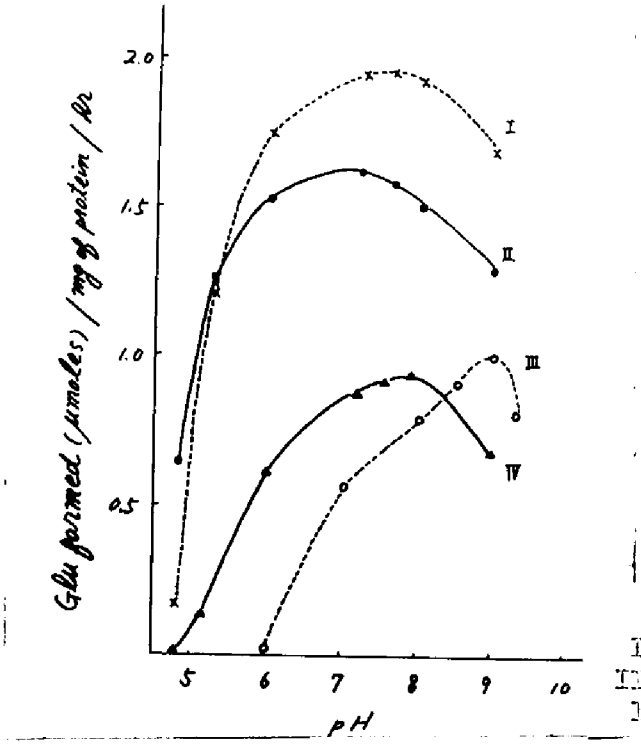



Effect of pH on transaminase activity:

Fig. IV-4 shows the effect of pH on transaminase activity of these four reaction systems. Borate buffer was employed in place of phosphate buffer when higher than pH 8.0. The optimal pH of γ-ABA — KGA, Asp — KGA, Orn — KGA and β-Ala — KGA transaminase systems were observed at about 7.5, 7.2, 9.0 and 7.8, respectively. It is noteworthy that the optimal pH of Orn — KGA transaminase was found to be more alkaline side than those of the other enzymes. The activity of Asp — KGA transaminase decreased slowly over the wide range of pH 6.0 ~ 8.0 and this fact coincides with the observation on the same reaction by the preparation of Fl. fuscum as

mentioned in the following chapter.

Fig. IV-4. Effect of pH on the transaminase activity.




I. YABA — KGA
II. Asp — KGA
III. Orn — KGA
IV. βAla — KGA

Effect of inhibitor on transaminase activity:

Table IV-5 shows the effect of inhibitors on the enzyme activity. Hydroxylamine gave the most inhibitory effect on Y-ABA — KGA and Asp — KGA systems, but influenced less on the other reactions.

Table IV-5. Effect of inhibitor on transaminase activity of Ach. superficialis

| Inhibitor or reactivator | concentration M | Degree of inhibition (%) Y-ABA-KGA | Asp—KGA | β-Ala—KGA | Orn—KGA |
|---|---|---|---|---|---|
| SC | $10^{-3}$ | 0 | 0 | 0 | 0 |
| TSC | $10^{-4}$ | 0 | 0 | 0 | 0 |
| " | $10^{-3}$ | 5 | 20 | 0 | 13 |
| INAH | $10^{-3}$ | 20 | 12 | 6 | 8 |
| PCMB | $10^{-5}$ | 0 | 0 | 0 | 0 |
| " | $10^{-4}$ | 100 | 40 | 21 | 100 |
| $10^{-4}$ +Cysteine | $10^{-3}$ | 0 | 18 | 0 | 15 |
| KCN | $10^{-3}$ | 0 | 0 | 0 | 0 |
| $NH_2OH$ | $10^{-4}$ | 74 | 84 | 45 | 61 |
| " $10^{-4}$ + PALP 40Y/ml | | 0 | 10 | 6 | 6 |
| EDTA | $10^{-3}$ | -10 | -8 | 0 | 0 |
| Nitroso-R salt | $10^{-3}$ | 0 | 0 | 0 | 0 |

Enzyme preparation and an inhibitor were preincubated at 37°C for 15 min. before the addition of substrates.

SC: Semicarbazide, TSC: Thiosemicarbazide, INAH: Isonicotic acid hydrazide, PCMB: p-Chloromercuribenzoate, EDTA: Ethylenediaminetetraacetate.

The loss of activity by hydroxylamine was partially recovered by the addition of PALP. Isonicotic acid hydroazide (INAH) showed weak inhibition on the four reactions, while semicarbazide, potassium cyanide and nitroso-R salt inhibited little or not at all these systems. p-Chloromercuribenzoate inhibited actively all of them, especially it produced remarkable inhibition on γ-ABA — KGA and Orn — KGA transaminase reactions, although the inhibition was recovered partially by the addition of cysteine. This fact suggests that SH group on the enzyme has an important role. Ethylenediaminetetraacetate (EDTA) showed no inhibition, but rather a little stimulative effect.

Effect of PALP:

The effect of pyridoxal phosphate on the activity of transaminase reaction of each fraction was demonstrated in Table IV-6. Each

Table IV-6. Effect of pyridoxal-5-phosphate on the activity of fractions.

| System | Asp — KGA | | γ-ABA — KGA | |
|---|---|---|---|---|
| PALP | None | Added | None | Added |
| Sonicate | 1.89 | 3.15 | 1.79 | 1.78 |
| protamine treatment | 2.85 | 5.33 | 3.38 | 3.38 |
| $(NH_4)_2SO_4$ 33 75 % | 1.65 | 7.65 | 4.35 | 4.30 |
| Alumina Cγ treatment | 0 | 11.85 | 15.30 | 15.00 |

fraction was dialyzed against 0.005 M phosphate buffer of pH 7.8 containing 0.01 % 2-mercaptoethanol for 5 hr at 4°C after each purifying procedure. Asp — KGA transaminase activity of dialyzed sonicate decreased to 60 % and the final preparation subjected to alumina gel Cγ showed no activity without the addition of PALP. Asp — KGA transaminase released so easily the coenzyme during the purification treatment and dialysis, as its activity was recovered by the addition of PALP, while γ-ABA — KGA transaminase could reveal the full activity without the addition of the cofactor throughout the procedure.

Transamination between taurine and KGA by the extract of Achromobacter superficialis:

All the amino donors known to participate in transamination have one or two amino groups and one or two carboxyl groups, or a carboxylamide group, e.g. in Glu($NH_2$) and Asp($NH_2$). However, it was found that taurine involving an amino group and a sulfonic acid group, but no carboxyl group could be transaminated to KGA to form Glu by the resting cells and extract of Ach. superficialis. The amount of taurine was estimated by circular paper chromatography and ninhydrin reaction as usual.

Table IV-7. Transamination between taurine and KGA in Ach. superficialis.

| Incubation time | 0 min | 30 min | 60 min |
|---|---|---|---|
| Taurine | 20 μmoles | 18 μmoles | 16.2 μmoles |
| KGA | 20 | | |
| Glu formed | 0 | 0.21 | 0.3 |
| Taurine | 20 | 19.4 | 19.2 |
| KGA | 0 | | |
| Glu formed | 0 | 0 | 0 |
| Taurine | 0 | 0 | 0 |
| KGA | 20 | | |
| Glu formed | 0 | 0.05 | 0.08 |

Taurine, KGA, PALP (20 μg), phosphate buffer (pH 7.8, 100 μmoles) and crude extract (protein 1mg), were presented in 1.0 ml. Incubation was carried out at 37°C.

As demonstrated in Table IV-7, the formation of Glu in the complete reaction proceeded stoichiometrically with the disappearance of taurine, although the taurine transaminase activity was relatively weak. In the control system lacking KGA or taurine Glu was formed not at all or very little. As shown in Table IV-8, the optimal pH of the taurine trans-

Table IV-8. Effect of pH on taurine — KGA reaction.

| pH | 5.2 | 6.3 | 7.0 | 7.8 | 7.8** | 8.2 |
|---|---|---|---|---|---|---|
| Activity* | 0 | 0 | 0.13 | 0.22 | 0.27 | 0.20 |

*: formed Glu (μmoles)/mg of protein/hr.
**: Tris-buffer, the rest represent phosphate buffer.

amination was observed at about 7.8 and when tris-buffer was used in place of phosphate buffer, a little higher activity was demonstrated.

A keto analogue of taurine was not detected by a usual procedure for ketonic acid.

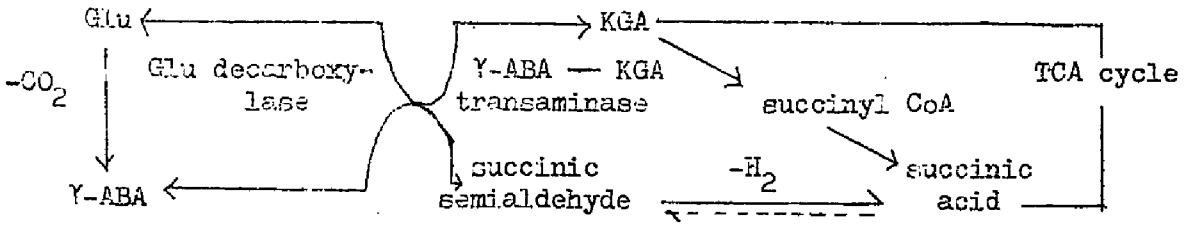
The main five transaminase reactions by the resting cells and extract of Ach. superficialis were summarized in Table IV-9. Among the active amino donors, four amino acids except Asp belong to ω-amino acid. The physiological importance of these ω-amino acid transaminases in amino acid metabolism of this organism has not yet elucidated. Transamination between γ-ABA and KGA, first reported by Bossmen and associates (42), represents another examples of ω-amino group transfer.

Table IV-9. Transaminase activities in Ach. superficialis.

| Amino group donors | | Activities | |
|---|---|---|---|
| | | Cells* | Extract** |
| L-Asp | $HOOC(CH_2)_2\underset{NH_2}{CH}COOH$ | 3.5 | 1.60 |
| L-Orn | $NH_2(CH_2)_3\underset{NH_2}{CH}COOH$ | 1.8 | 0.90 |
| γ-ABA | $NH_2(CH_2)_3COOH$ | 3.3 | 1.80 |
| β-Ala | $NH_2(CH_2)_2COOH$ | 1.8 | 0.60 |
| Taurine | $NH_2(CH_2)_2SO_3H$ | 0.7 | 0.22 |

*: ΔO formed Glu (μmoles)/mg. of dry wt./hr
**: formed Glu (μmoles)/mg. of protein/hr

In addition to γ-ABA (43), there is good evidence that β-Ala (42, 23), δ-aminovaleric acid (44,45, 46) and α,γ-diaminoglutaric acid (44) also participate in transamination. As a result of studies in detail on the γ-ABA — KGA reaction, it seems difficult merely to regard γ-ABA as an end-product of the decarboxylation of Glu, and, therefore, it is required to consider alternative pathways by which γ-ABA may arise and be utilized. Steward and Pollard (47) suggested the scope of these possible relationships, i.e. a coupled reaction of Glu decarboxylation and γ-ABA — KGA transamination as follows:




However, with Ach. superficialis employed here, no activity of Glu decarboxylase was found by both the resting cells and the extract and, therefore, it is difficult to suggest that there is a so-called γ-ABA cycle proposed in such bacteria as E. coli for this bacterium.

D-Amino acid transaminase:

In early studies, it was reported by some workers that certain D-amino acids could participate in transamination, while later consideration and repetition of these experiments led to the conclusion that D-amino acids were inactive. The recent studies of Thorne and his associates (48, 49, 50) provided convincing evidence for the participation of D-amino acids in transamination reactions catalyzed by certain bacteria which synthesize extracellular polyglutamic acid predominantly of the D-configuration. They found that cell-free preparations of Bacillus subtilis catalyzed the formation of D-Glu from KGA and D-Ala, D-Asp, and certain other D-amino acids. Preparations of Bacillus anthracis catalyzed transamination between PyA and the D-isomers of Phe, Try, Met, His and Leu to yield D-Ala.

An investigation of the synthesis of D-Ala in Streptococcus faecalis by Wood and Gunsalus (51) resulted in the discovery that this bacterium possessed an enzyme system capable of catalyzing the formation of racemic Ala from either D- or L- Ala.

The formation of Glu from L-Ala or D-Ala and KGA and the reverse reaction, i.e. the formation of Ala from L-Glu or D-Glu and PyA by the resting cells and extracts of several bacteria were investigated.

Table IV-10. Transamination of D-amino acid by resting cells

| | Reaction system | I | | II | |
|---|---|---|---|---|---|
| | | Ps. fluorescens | B. thiaminolyticus | B. natto SN | B. roseus |
| formed Glu | L-Ala + KGA | 3.3 | 2.1 | 6.0 | 5.5 |
| | D-Ala + KGA | 3.4 | 2.2 | 6.0 | 6.5 |
| formed Glu | L-Glu + Pyr | 3.6 | 2.6 | 0 | trace |
| | D-Glu + Pyr | 0 | 0 | 6.1 | 6.0 |

As shown in Table IV-10, Glu was actively produced from KGA and either L-Ala or D-Ala by the resting cells of Ps. fluorescens, B. thiaminolyticus, B. natto SN and B. roseus. The formation of Ala was observed in the reaction systems containing PyA, L-Glu, and the resting cells of either Ps. fluorescens or B. thiaminolyticus, while Ala was not formed at all from D-Glu and PyA. On the contrary, in the presence of the resting cells of B. natto SN and B. roseus, Ala was not produced from L-Glu and PyA, but formed from D-Glu and PyA at the nearly same rate as that of Glu formation. These observations suggest that L-Glu — PyA (or L-Ala — KGA) transamination reaction occurs and the

reaction involving the D-isomer of Glu is not found in the cells of Ps. fluorescens and B. thiaminolyticus, while there is an active D-Glu — PyA (or D-Ala — KGA) transamination reaction, but no L-Glu — PyA reaction in the other two strains. The fact that Glu was produced from KGA and either L-Ala or D-Ala at the same degree in these four bacteria, suggests that the resting cells of these strains involve Ala-racemase and D-Ala was used as an amino donor after it was partially converted to the L-isomer by Ala racemase in the resting cells of Ps. fluorescens and B. thiaminolyticus, and L-Ala was racemized also by the racemase and the resulting D-isomer was transaminated with KGA by D-Ala — KGA transaminase in the case of B. natto SN and B. roseus. The evidence for the existence of Glu racemase was not presented in all the bacteria. If Glu racemase should present, Ala would be formed from PyA and either L-Glu or D-Glu. The results of these observations and suggestions were summarized in Schema IV-1 and Table IV-11.

Schema IV-1. Metabolism of L- and D-Ala, and L- and D-Glu.

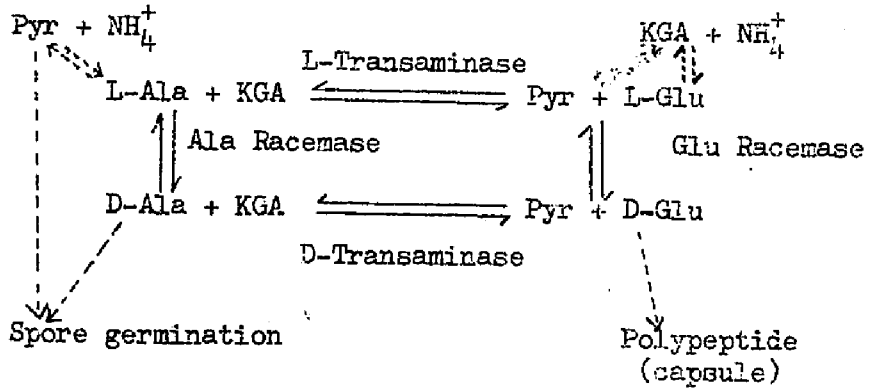



Table IV-11. The distribution of activity of enzymes relating Ala metabolism

| Enzyme system | I | | II | |
|---|---|---|---|---|
| | Ps. fluorescens | B. thiaminolyticus | B. natto SN | B. roseus |
| L-Transaminase | + | + | - | - |
| D-Transaminase | - | - | + | + |
| Ala Racemase | + | + | + | + |
| Glu Racemase | - | - | - | - |

But, some amount of Ala was produced from PyA and D-Glu by the cell-free extract of Ps. fluorescens and the permeability of cell membrane might have to be considered to elucidate the observations above.

The high activities of transaminase reaction involving Ala, and

Ala racemase have been postulated to be essential or at least important in the germination of spores in bacilli (52). Table IV-12 shows the activities of transaminase reactions of the cell-free extracts of Cl. acetobutylicum, an anaerobic bacterium forming spores, and B. roseus, an aerobic bacterium forming spores. It is interesting that in the

Table IV-12. Transaminase activities of the cell-free extract of Clostridium acetobutylicum W29 and Bacillus roseus.

| | Activity (μmoles of Glu formed /mg protein/hr) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Amino donor | L-α-Ala | D-α-Ala | β-Ala | L-Asp | L-Leu | L-Met | L-Phe | L-Val |
| Cl.acetobutylicum | 0 | 0 | 0 | trace | 2.7 | 1.0 | 0.7 | 2.3 |
| B. roseus | 3.2 | 3.1 | 0 | 2.5 | 0 | 0 | trace | trace |

extract of Cl. acetobutylicum, L-Ala, D-Ala and Asp were hardly transaminated with KGA and Leu — KGA, Met — KGA, Phe — KGA and Val— KGA transaminase reactions were actively observed, while in the case of the preparation of B. roseus, L-Ala—KGA, D-Ala — KGA and Asp — KGA reactions were very active and Leu, Met, Phe and Val were little or not at all transaminated with KGA. The transamination of the extract of Cl. acetobutylicum is unique to reveal a trace of Asp — KGA transaminase activity which were distributed generally among all the bacteria investigated here.

## Summary


1. Transaminases of the cell-free extract of Fl. fuscum were fractionated and their properties were investigated.

a. Several active transaminations observed may be catalyzed by some different enzymes.

3. Four main reactions observed in the preparation of Ach. superficialis, i.e. Asp —, γ-ABA —, β-Ala — and Orn — KGA transamination were also suggested to be catalyzed by each different enzyme.

4. γ-ABA — KGA apotransaminase was firmly combined with the coenzyme during the purification processes, while Asp — KGA transaminase seems to release easily the cofactor, PALP, under the same condition.

5. Taurine involving an ω-amino group and sulfonic acid group, could participate in transamination with KGA by the extract of Ach. superficialis. The physiological importance of ω-amino acid transamination was discussed.

6. L-Ala — KGA (or L-Glu — PyA) transaminase activity occurred in Ps. fluorescens, and B. thiaminolyticus lacking D-Ala — KGA (or D-

Glu — PyA) transaminase activity, while D-Ala — KGA (or D-Glu — PyA) transaminase activity was found in B. natto SN and B. roseus having no activity of L-Ala transaminase.

7. In these four bacteria, there is generally Ala racemase, but Glu racemase reaction was not found.

8. The transamination of Cl. acetobutylicum is unique to show a faint Asp — KGA transaminase activity which is generally distributed in bacteria and no activity of either L-Ala or D-Ala transaminase.

## V. Transaminase Reactions in Fungi

With the exception of problems in biosynthesis of certain amino acids, e.g. Cys and Lys, studies on amino acid metabolism of the fungi have been relatively neglected. Numerous surveys of nitrogen nutrition have been made, unfortunately too often without a clear conception of the physiological problems involved. Little is known of the mechanism of nitrogen assimilation into amino acids and proteins, and the metabolism of amino acids, especially the transamination reaction.

Only a few works on the transamination of fungi have been reported hitherto. Michi et al. (53) briefly reported the formation of Glu from Ala or Asp and KGA by the transaminase of extracts of Aspergillus or Penicillium. Kategiri and Tochikura (28) investigated the distribution of the kinds of transamination between KGA and Asp, Leu , or Ileu, in some fungi. Roberts et al. (54) observed that Aspergillus fumigatus homogenates catalyzed the transamination between KGA and Y-ABA to form Glu and succinic semialdehyde. This represents the occurrence of the similar transfer of an ω-amino group in fungi as mentioned above in Achromobacter superficialis (35, 36) and is similar to the formation of Glu from Orn and KGA by the extracts of Neurospora crassa (22, 55).

A kynurenine transaminase catalyzing the transfer of the α-amino group of kynurenine or 3-hydroxykynurenine to keto acids, was partially purified from the extracts of Neurospora crassa (56).

Wagner et al.(57, 58) observed the transamination reaction between Phe, Val or Ileu and such ketonic acids as KGA, α-keto-β-methylvaleric acid or α-ketoisovaleric acid by the extract of Neurospora crassa and purified partially these enzymes and investigated the kinetic behavior of PPA transaminase.

In the present chapter, the distribution and the kinds of transaminase activities of the cell-free extracts of several strains of Aspergillus, Penicillium, Rhizopus, Mucor, Monascus and Neurospora were surveyed and some properties of transaminases of Aspergillus oryzae and the effect of nitrogen sources of growth media on the enzyme activities were also investigated.

### Materials and Methods

**Microorganisms:**

The fungi employed in the experiments, unless otherwise stated, were cultivated in a medium prepared as follows: 3.0 % glucose, 0.4 % $NH_4NO_3$, 0.1 % $KH_2PO_4$, 0.2 % $K_2HPO_4$, 0.02 % $MgSO_4 \cdot 7H_2O$, 0.1 % peptone in tap water. The final pH was about 5.8. For the purpose of investigating the effect of nitrogen source of the medium on the transaminase activities, the following medium was employed. 2.0 % glucose, 0.1 % $KH_2PO_4$, 0.2 % $K_2HPO_4$, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.01 % peptone, plus 20 mmoles of a nitrogen source per 100 ml of a medium in tap water. The final pH was about 5.8.

One hundred ml of the culture medium in a 500 ml flask was incubated at 30 °C for 48~72 hr. on a shaker. The mycelia harvested by centrifugation were washed twice with water and the washed mycelia were stored at -20°C till being used.

The cell-free extract:

The deeply frozen mycelia were immediately ground with suitable amount of glass powder and 0.1 M phosphate buffer solution of pH 7.4 containing 0.01 % 2-mercaptoethanol, at 4 °C. The debris and glass powder were removed by centrifugation at 11,000 r.p.m. for 30 min, and the resulting supernatant was used as a crude cell-free extract.

Assay of transaminase activity:

The standard reaction system consisted of 40 μmoles of amino acid with the exception of 10 μmoles of Tyr, 40 μmoles of KGA, 20 μg of PALP, enzyme, 100 μmoles of phosphate buffer of pH 7.8. When DL-amino acid was used, 80 μmoles was added to the mixture.

Incubation was carried out at 37°C for 60 min. The amount of Glu formed and the residual amino donor were determined as described in Method of chapter II.

## Results and Discussion

Distribution of transaminase activity of extracts of mycelia:

(i) Aspergillus, Penicillium and Rhizopus.

Transaminase activity of the cell-free extracts of mycelia of two strains of Aspergillus, two strains of Penicillium, one strain of Rhizopus was assayed. Results on the distribution and the kind of the reaction between KGA and various amino acids were illustrated in Table V-1. Transaminations with Ala, Asp, Leu and Phe were generally observed all these strains and above all it is interesting that there was a very active α-Ala — KGA transaminase reaction, which was found

Table V-1. Transaminase activity of extracts from mycelium

| amino group donor | Activity(formed Glu(μmoles)/mg/hr) Asp. oryzae | Asp. tamarii | Pen.chrysogenum | Pen. expansum | Rh.** batatus |
|---|---|---|---|---|---|
| DL-α-ABA | 0.56 | 0.35 | 0.14 | 0 | 0 |
| Y-ABA | 0 | 0 | 0 | 0 | 0.54 |
| L-α-Ala | 3.62 | 0.75 | 1.13 | 3.75 | 0.53 |
| β-Ala | 0 | 0 | 0 | 0 | 0 |
| L-Asp | 2.20 | 3.76 | 1.03 | 4.20 | 4.00 |
| L-Leu | 0.56 | 0.40 | 0.38 | 2.40 | 0.55 |
| L-Lys | 0 | 0 | 0 | 0 | 0 |
| L-Met | 0 | 0 | 0 | 0 | 0 |
| L-Orn | 0 | 0 | 0 | 0 | 2.00 |
| L-Phe | 0.73 | 0.35 | 0.33 | 3.30 | 0.53 |
| L-Tyr* | 0.68 | 0 | 0 | 2.42 | 0.53 |
| L-Try | 0.83 | 0 | 0 | 2.81 | 0 |
| L-Val | 0 | 0.30 | 0.18 | 2.30 | 0.95 |

L-Amino acid (40 μmoles), KGA (40 μmoles), PALP (20Y), enzyme, pH 7.8 phosphate buffer(200 μmoles), at 37°C.
*: 10 μmoles, **: extract from dried mycelium.

in the relatively limited genera in bacteria, e.g. Bacillus, Proteus, Pseudomonas and Corynebacterium. Preparations revealing α-Ala — KGA transaminase activity was accompanied with that of α-ABA — KGA transamination reaction in those strains of bacteria, however, in Penicillium expansum and Rhizopus batatus, α-Ala was used as a relatively active amino donor, while α-ABA was not at all. Although Roberts et al. (54) reported the occurrence of Y-ABA — KGA transamination in the extract of Aspergillus fumigatus, such a reaction was not observed in the preparation of two strains of Aspergillus employed in this experiment. β-Ala, Lys and Met were not transaminated with KGA in these fungi.

Transaminase activity was hardly found in the cell-free extract of the intact mycelia of Rhizopus batatus, while the fair activity was observed in the extract from the dried mycelia. The transaminase might not be extracted from the intact mycelia by the grinding with glass powder, but rather easily released from mycelia after being dried enough in the air, probably because of the destruction of the fine structure of cell wall.

(ii) Mucor, Monascus and Neurospora.

The distribution and kinds of the transaminase activity in two strains of Mucor, one strain of Monascus, and two strains of Neurospora

were listed in Table V-2.

Table V-2. Transaminase activity of extracts from mycelium

| Amino group donor | Activity | | | | |
|---|---|---|---|---|---|
| | Mucor rouxii | Mu. circinelloides | Monascus anka | Neurospora crassa | N. sitophila |
| DL-α-ABA | 0 | 0 | 0.19 | 0.50 | 0 |
| Y-ABA | 1.50 | 1.05 | 0.23 | 0.45 | trace |
| L-α-Ala | 0 | 0 | 0.30 | 0.90 | 0 |
| β-Ala | 0 | 0 | 0.60 | 0 | 0 |
| L-Asp | 2.00 | 2.40 | 2.32 | 2.32 | 0.98 |
| L-Leu | 0.80 | 0.54 | 0.82 | 1.11 | 1.62 |
| L-Lys | 0 | 0 | 0 | 0 | 0 |
| L-Met | 0 | 0 | 0.81 | 0.45 | 1.32 |
| L-Orn | 0 | 0.67 | 0 | 0.45 | 0 |
| L-Phe | 0.51 | 0 | 1.27 | 0.60 | 0.85 |
| L-Try | 0 | 0 | 0.23 | 0 | 0 |
| L-Tyr | 0 | 0.83 | 0.40 | 0.40 | 0 |
| L-Val | 0 | 0 | 0.50 | 0.75 | 0 |

In two strains of Mucor, it is interesting that α-Ala — KGA and α-ABA — KGA reaction systems did not occur, and especially in the extract of Mucor circinelloides, Phe was not also transaminated with KGA. On the other hand, Y-ABA was highly utilized as an amino donor.

In the cell-free extract of Monascus anka, various amino acids except Lys and Orn, were transaminated with KGA. In the case of the extract of Neurospora crassa, also many amino acids could participate in transamination, however, in Neurospora sitophila only four amino acids were used as active amino donors and even Ala — KGA reaction was not found as well as in Mucor.

In these fungi, Asp, Leu and Phe were active amino donors to KGA. Such distribution is near that of various bacteria except Ach. superficialis, Ach. polymorph and Agrobacterium tumefaciens. In the fungi employed in this experiment except two strains of Mucor, and Neurospora sitophila, L-Ala was actively transaminated with KGA to form Glu, but D-Ala did not replace the L-isomer, although D-Ala was transaminated with KGA as well as L-Ala in Bacillus and Pseudomonas. Ala was formed from L-Glu and PyA, but not from D-Glu and PyA by the extracts of the fungi employed. This fact suggested that there is neither Ala racemase nor Glu racemase, both of which are regarded as a key-reaction for spore germination as well as D-Ala transaminase and Ala dehydrogenase (52).

Effect of nitrogen source of the growth media on the activity:

The transaminase activity of extracts from mycelia grown in synthetic media involving various nitrogen sources was determined and the change of the activity owing to nitrogen source was listed in Table V-1.

Table V-1. Effect of nitrogen sources in the medium on the activity. (Pseudomonas oxyans)

| | Activity of extracts from mycelium in the various media | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N-source | Peptone* | peptone | $NH_4NO_3$ | Ala | Glu* | Asp | γ-ABA | Phe | Leu | Urea | Asp($NH_4$)$_2$ | Val |
| DL-α-ABA | 0.56 | 0.65 | 0 | 0 | 0 | 0 | 0 | tr | 0 | 0 | 0 | 0 |
| γ-ABA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L-α-Ala | 3.62 | 3.85 | 2.05 | 1.27 | 1.81 | 0.63 | 1.65 | 4.73 | 1.70 | 4.2 | 1.40 | 1.3 |
| β-Ala | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L-Asp | 2.20 | 2.35 | 1.77 | 2.00 | 2.41 | 1.52 | 1.44 | 4.70 | 2.22 | 3.46 | 1.81 | 2.01 |
| L-Leu | 0.56 | 1.57 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L-Lys | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L-Met | 0 | 0 | 0.39 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L-Orn | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L-Phe | 0.73 | 1.54 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.30 | 0.31 | 0 |
| L-Try | 0.83 | 0.40 | 0 | 0 | 0 | 0 | 0 | 0 | 0.51 | 0 | 0 | 0 |
| L-Tyr | 0.68 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L-Val | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*: $CaCO_3$ free.

Extract from mycelia grown in the medium involving peptone as nitrogen source either with or without the addition of $CaCO_3$ showed the distribution of many reaction systems and generally high activity for each system. Although Phe and urea gave a promoting effect on the transaminase activity in the Ala — KGA and Asp — KGA systems, the rest of the synthetic media showed no marked influence.

The kinds of reactions observed were hardly changed according to the kinds of nitrogen sources of growth media and it is not likely that these enzymes were inducible from the point of view of the effect of nitrogen sources.

Effect of age of mycelium on the transaminase activity:

Table V-4 shows the relationship between transaminase activity and age of mycelium of Aspergillus oryzae grown in the medium described in the paragraph of Methods. Activities of α-ABA — KGA, Leu — KGA and Phe — KGA transaminase reactions disappeared almost after about 36 hr. of the growth.

Table V-4. Transaminase activity and age of mycelium (Asp. oryzae)

| | Activity | | | | | |
|---|---|---|---|---|---|---|
| Age(hr) / Amino donor | 24 | 36 | 48 | 72 | 84 | 120 |
| DL-α-ABA | 0.25 | 0 | 0 | 0 | 0 | 0 |
| L-α-Ala | 3.02 | 3.25 | 2.04 | 1.34 | 1.40 | 1.63 |
| L-Asp | 3.14 | 3.02 | 2.87 | 2.60 | 2.70 | 2.75 |
| L-Leu | 0.35 | 0 | 0 | 0 | 0 | 0 |
| L-Phe | 0.65 | 0 | 0 | 0 | 0 | 0 |

Ala transaminase and Asp transaminase maintained their activities during the long periods of incubation, although they decreased gradually according to the age of mycelium. Asp transaminase, especially, changed scarcely the activity and could reveal a relatively high activity during the long periods of growth. This fact suggests that these two transaminase reactions have important roles in amino acid metabolism of the fungi employed.

Effect of detergent on extraction of transaminase:

Various surface active detergents have been known to give solubilization effect on protein (59), and Katagiri and Ikemiya (60) carried out the selective determination of α- and β-amylases by means of detergent and recently Negoro (61) reported that some detergents could be utilized as a useful agent to prepare crystaline enzymes, e.g. saccharase, amylase and protease.

In order to investigate the effect of detergents on the extraction of transaminase from mycelium of Asp. oryzae, mycelia ground with glass powder and phosphate buffer as described above, were mixed with sodium dodecyl sulfate (an anionic detergent) or Lauryl pyridinium chloride (a cationic detergent), and glass powder and cell debris were removed by centrifugation after the mixture was incubated on a shaker at 30°C for 10 min.

The supernatant was dialyzed against running water at 14°C for

1 hr and then dialyzed against 0.005 M phosphate buffer of pH 7.3 with stirring at 4°C for 4 hr to remove the detergent. The transaminase activity and the amount of protein of the resulting solution were determined. As shown in Table V-5, the amount of extracted

Table V-5. Effect of detergents on extraction of transaminase

| Detergent | Extracted protein (mg) | Specific activity, Ala— KGA | Asp— KGA | Total activity, Ala— KGA | Asp— KGA |
|---|---|---|---|---|---|
| None | 47 | 2.07 | 2.37 | 95 | 135 |
| SDS ($10^{-2}$M) | 110 | 1.73 | 2.82 | 1.84 | 3.10 |
| LPC ($10^{-2}$M) | 26 | 1.09 | 3.92 | 28 | 102 |

SDS: Sodium dodecyl sulfate
LPC: Lauryl pyridinium chloride

protein increased more than two fold, although the specific activity of Asp — KGA transaminase decreased a little and that of Ala — KGA transaminase changed hardly by the addition of sodium dodecyl sulfate and as a result, the total activity of both transaminase reactions increased. Especially, Asp — KGA transaminase could be remarkably extracted from mycelium without loss of the activity by means of this detergent.

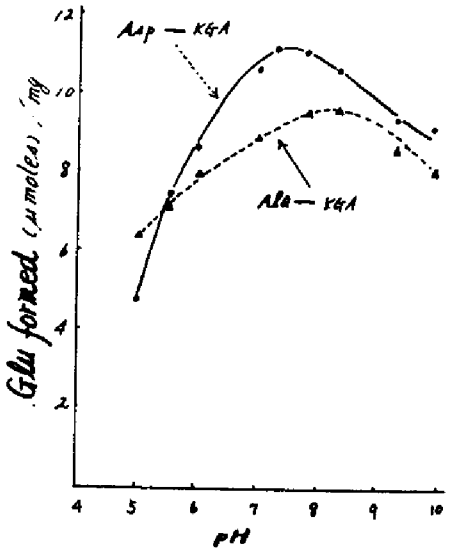
When lauryl pyridinium chloride was added to the mixture, the specific activity of Ala — KGA transaminase fell remarkably and a little total activity was obtained, however, the specific activity of Asp — KGA transaminase increased somewhat. The Asp — KGA transaminase seems to be more stable than the other transaminases against the change of growth period and the addition of detergents.

As the effective procedure of extraction of enzymes from mycelia of fungi without loss of activity has not known as compared with a convenient treatment for the bacterial cells, e.g. sonic disruption, it is likely that such detergents might be utilized as a useful agent for the extraction of the other enzymes from mycelia in a stable state.

**Effect of pH on activity:**

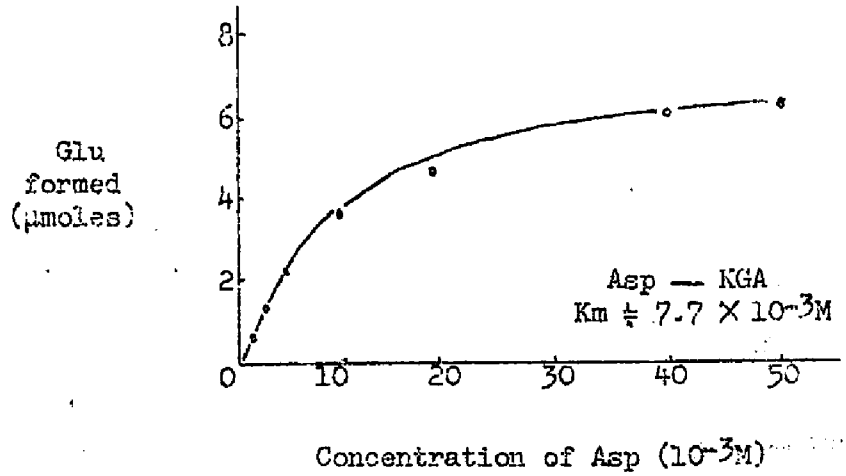
Fig. V-1 shows the effect of pH on the activities of Ala — KGA and Asp — KGA transaminases of Aspergillus oryzae. The optimal pH for Asp — KGA and Ala — KGA reaction systems was about 7.2 and 7.8, respectively. In the former, the activity fell sharply in the acidic

Fig. V-1. Effect of pH on the transaminase activity




Borate buffer was used above pH 8.0.

Fig. V-2. Effect of concentration of Asp on the transaminase activity.

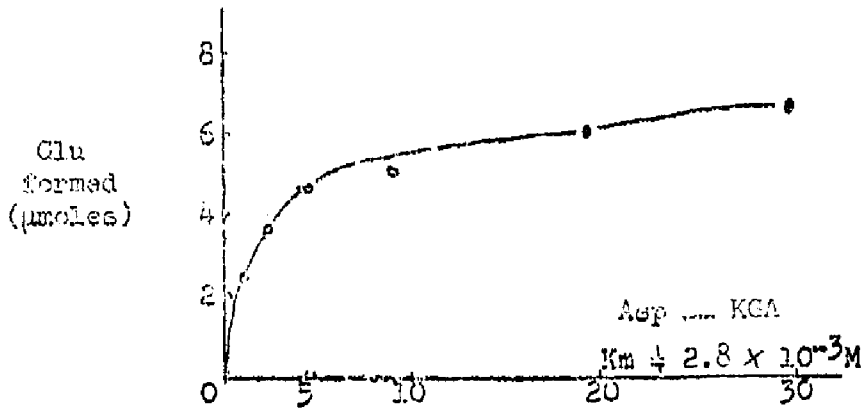



side of the optimal pH and the decrease of it progressed slowly at the more alkaline pH.

Effect of concentration of substrate on activity:

In order to determine the Michaelis constant (Km) for Asp and KGA in the transaminase system of Aspergillus oryzae, sufficient amount of the other substrate and PALP were added to ensure that the enzyme system is saturated and that the only material influencing

Fig. V-3. Effect of concentration of KGA on the transaminase activity.




the rate of the reaction is the substrate under the test conditions. Relations between concentration of Asp or KGA and the activity were illustrated in Fig. V-2 and V-3 and were plotted graphically according to the method of Lineweaver-Burk (62). The Km values obtained for Asp and KGA were about $7.7 \times 10^{-3}$M and $2.8 \times 10^{-3}$M, respectively. These values are not so different from those for several transaminase reactions in the preparation of Fl. fuscum as mentioned in the following chapter.

## Summary

1. In the extracts of strains belonging to Aspergillus, Penicillium and Rhizopus, transaminase reactions between KGA and Ala, Asp, Leu and Phe were generally active.

2. Transaminase of Rhizopus was easily extracted not from the intact mycelia, but from the dried mycelia.

3. In the extract of Mucor, Ala — KGA and α-ABA — KGA transaminase activities were faint, but Y-ABA — KGA reaction was very active.

4. In the extract of Monascus and Neurospora crassa, various amino acids were utilized as an amino donor to KGA, but in the extract of Nerospora sitophila, only four transaminase reactions were found.

5. In the fungi employed in these experiments except two strains of Mucor, and Neurospora sitophila, L-Ala was uniformly used as an active amino donor with KGA, but D-Ala transaminase did not occur.

6. The kinds and activities of transaminases of these fungi were not so changed according to the kind of nitrogen source of the growth media.

7. Asp — KGA transaminase activity of Aspergillus oryzae changed hardly and revealed a relatively high activity during the long periods of growth.

8. Asp — KGA transaminase of Aspergillus oryzae was efficiently extracted from mycelia without loss of the activity by the addition of sodium dodecyl sulfate.

9. Effects of pH and substrate concentration on the transaminase activity were investigated.

## VI- Transamination in Actinomycetales, Fungi Imperfecti and Yeasts.

The distribution of transaminase activity in fungi imperfecti, Actinomycetales and yeasts is interesting when it is compared with those in bacteria and fungi from the standpoint of comparative biochemistry, however, little work on this line has been presented. Transamination in fungi imperfecti has not yet studied at all and the existence of Asp — KGA transaminase activity in the extract of Streptomyces fradiae was briefly reported by Romano and Nickerson (63).

In yeasts, transamination reactions have been demonstrated in only a few strains; Torulopsis utilis (64), Saccharomyces fragilis (65), brewer's yeast (66), Saccharomyces rouxii major (67) and Saccharomyces cerevisiae (68) and these studies were directed to the limited genera of yeast.

In the present chapter, transaminase activities of cell-free extracts from fungi imperfecti and five strains belonging to various genera of yeasts were demonstrated and the characteristic of the distribution and the kinds of transaminase reactions were discussed.

### Materials and Methods

Microorganisms:

Gibberela fujikuroi (Sawada) Wr., Fusarium lini Bolley, and Pullularia pullulans (de Bary et Berkhout) were grown in the same media and under the same conditions as stated in the methods of the previous chapter.

Streptomyces ruber and Actinomyces asteroides were cultured in the medium prepared as follows; soluble starch 1.0 %, glucose 1.0 %, bouillon 0.3 %, yeast extract 0.3 %, $(NH_4)_2SO_4$ 0.5 %, $KH_2PO_4$ 0.1 %, $K_2HPO_4$ 0.2 %, in tap water. The final pH was about 7.0. Culture was carried out on shaker at 30°C for 48 ~ 72 hr. The cells harvested by centrifugation were washed twice with 0.85 % NaCl solution and suspended into 0.1 M phosphate buffer of pH 7.8.

Five strains of yeasts were grown in the medium composed of Koji extract (8° Bllg) and 1 % lactate. The final pH was about 6.0. Incubation was carried out on shaker at 30°C for 48 ~ 72 hr. The cells harvested by centrifugation were twice washed with 0.85 % NaCl solution and suspended into 0.1 M phosphate buffer of pH 7.8.

Cell-free extracts:

The mycelia of Gibberela fujikuroi and Fusarium lini were ground with glass powder and 0.1 M phosphate buffer of pH 7.8 as mentioned in the methods of the previous chapter.

The washed cells of the other microorganisms were treated in Ratheon sonic oscillator, 10 kc , for 20 min and cell debris was removed by centrifugation at 11,000 r.p.m. for 30 min.

The resulting supernatant was dialyzed against 0.005 M phosphate buffer of pH 7.8 containing 0.01 % 2-mercaptoethanol for 15 hr at 4°C. The solution obtained was used as an enzyme solution in the case of fungi imperfecti, Actinomycetes, Streptomyces and Zygosaccharomyces major. The extracts of the other yeasts were stored at -20°C for 15 hr and after thawing of the frozen solution, the precipitated residue was removed by centrifugation. The resulting clear solution was employed to assay the transaminase activity

Assay of transaminase activity:

Assay of the activities of transaminases in these preparations was done as mentioned in the paragraph of the methods of the previous chapter.

## Results and Discussion

As shown in Table VI-1, the distribution and kinds of transaminase activities in 3 strains of fungi imperfecti, Gibberela fujikuroi, Fusarium lini and Pullularia pullulans, were similar to those of the

Table VI-1. Transaminase activity of extracts from mycelium

| Amino group donor | Activity | | | | |
|---|---|---|---|---|---|
| | Gibberella fujikuroi | Fusarium lini | Pullularia pullulans | Streptom. ruber | Actinom. asteroides |
| DL-α-ABA | 0 | 0 | 0.42 | trace | 1.25 |
| Y-ABA | 0 | 0 | 0 | 0.63 | 0 |
| L-α-Ala | 2.61 | 0.36 | 0.45 | 0 | 0 |
| β-Ala | 0 | 0 | 0 | 0 | 0 |
| L-Asp | 1.90 | 0.46 | 1.86 | 0.84 | 3.43 |
| L-Leu | 0.30 | 0 | 0.70 | trace | 1.00 |
| L-Lys | 0 | 0 | 0 | 0 | 0 |
| L-Met | 0 | 0 | trace | 0 | 0 |
| L-Orn | 0.34 | 0 | 0 | 0.41 | 0 |
| L-Phe | 0.38 | 0 | 0.62 | 1.44 | 0 |
| L-Try | 0 | 0 | 0 | 1.30 | 0 |
| L-tyr | 0 | 0 | 0 | trace | 0 |
| L-Val | 0 | 0 | 0.47 | 0 | 0.82 |

fungi belonging to Aspergillus as described above, i.e. the high activities of Ala — KGA and Asp — KGA transaminases occurred generally although in the case of the extract of Fusarium lini, the transaminase activity was faint for any reaction system.

In the extracts of Streptomyces ruber and Actinomyces astercides, the existence of Ala — KGA transaminase reaction was never observed, but in the latter case, it is interesting to point out that α-ABA — KGA and Leu — KGA reaction systems occurred rather actively.

In the extracts of yeasts, Asp, Leu, Met, Phe, Try, Tyr and Val were generally transaminated with KGA, while α-Ala, β-Ala, Lys and His did not participate in the transamination at all (Table VI-2).

Table VI-2. Transaminase activities of cell-free sonicate of yeast.

| Amino donor | Candida* rubra | Sacch.* sake No. 7 | Debary.* japonicus | Zygos. major | End.* lindneri |
|---|---|---|---|---|---|
| DL-α-ABA | 0 | 0 | 0 | 2.0 | 0 |
| γ-ABA | 1.33 | 0 | 1.67 | 0 | 0 |
| L-α-Ala | 0 | 0 | 0 | 0 | 0 |
| β-Ala | 0 | 0 | 0 | 0 | 0 |
| L-Asp | 10.30 | 6.20 | 4.00 | 0.42 | 1.60 |
| L-Leu | 3.30 | 3.14 | 6.75 | 1.34 | 10.00 |
| L-Lys | 0 | 0 | 0 | 0 | 0 |
| L-Met | 1.88 | 2.04 | 5.57 | 0.58 | 7.80 |
| L-Orn | 2.68 | 0 | 0 | 0 | 0 |
| L-Phe | 4.82 | 2.59 | 4.00 | 0.52 | 6.30 |
| L-His | 0 | 0 | 0 | 0 | 0 |
| L-Try | 4.46 | 1.57 | 3.60 | 0 | 5.58 |
| L-Tyr** | 2.23 | 0.80 | 2.20 | 0.46 | 2.10 |
| L-Val | 3.34 | 2.22 | 1.67 | 1.63 | 2.00 |

*: The precipitate was removed after freezing at -20°C for 24 hr.
**: 5 μmoles per ml.

The general distribution and the kind of transamination of several strains of yeasts demonstrated here resembls those of transamination in the Hughes press extracts of Saccharomyces fragilis reported by Bigger-Gehring (65), with the exception of existence of Ala — KGA transaminase activity in the strain.

It is interesting that Tyr — KGA transaminase activity occurred highly in the extracts of all the strains employed in the present experiment. The presence of this transaminase reaction might have

relations with the formation of higher alcohol from Tyr, i.e. tyrosol, a well-known component in many alcoholic fermentation products, as suggested by Sentheshanmuganathan (68). It is also noteworthy that besides Tyr, the other aromatic amino acids, Try and Phe, were actively transaminated with KGA in the presence of the extracts of yeasts.

VII. Lys — KGA transaminase reaction.

L-Lys is an indispensable constituent of the diet for all animals and there are much works on its metabolism in animals, plants and microorganisms. But, the metabolism of Lys is complicated and seems to remain relatively obscure as reviewed by Work (69).

Studies on the biosynthesis of Lys in E. coli (70, 71, 72), Neurospora crassa (73, 74, 75) and Torulopsis utilis (76), have provided evidence for existence of two different synthetic pathways; one is that via α,ε-diaminopimelic acid and the other via α-aminoadipic acid. Although it was found that L-Lys was degraded through pipecolic acid and α-aminoadipic acid to glutaric acid (77, 78), the first step of degradation, i.e. deamination process, has not yet been elucidated.

It has been postulated that the deamination of Lys might occur by oxidation or transamination reaction. L- or D- Lys is oxidized very faintly or not at all by L- or D- amino acid oxidase (79, 80, 81).

Transaminase reaction of Lys was studied in bacteria by Feldman and Gunsalus (13), and in plants by Wilson, King and Burris (82), and in animals by Cammarata and Cohn (83), and Awapara and Seale (84). But, very little or no transamination between L-Lys and KGA was observed in these experiments. The absence or the trace of the transaminase activity has been regarded as a feature of Lys metabolism.

As reported by the author previously (35, 36), during the course of the studies of transaminase activities in various bacteria, it was observed that Glu was formed from L-Lys and KGA by the resting cells or cell-free extracts of Fl. fuscum, Fl. flavescens and Ach. liquidum.

The present chapter deals with the partial purification and investigation of properties of the transaminase and then suggests that the keto analogue transaminated from Lys may exist as a ring form, dehydropipecolic acid.

## Materials and Methods

Microbiological methods:

Flavobacterium fuscum (Zimmermann, Bergy et al.), Fl. flavescens (Pohl), Achromobacter liquidum (Fr. et Fr.), Ach. superficialis, Ach. polymorph and Alcaligenes faecalis were employed in the present

chapter. They were grown in a liquid medium prepared as follows; 15.0 g peptone; 0.5 g $KH_2PO_4$; 1.0 g $K_2HPO_4$; 0.3 g $MgSO_4 \cdot 7H_2O$ and 2.0 g NaCl were dissolved in tap water and filled up to 1 liter. The final pH was about 7.2. Cultures were carried out with 100 ml medium placed in 500-ml flacks and kept on a shaker for 24 hr at 30°C. The cells were harvested and washed twice with 0.85 % sodium chloride solution.

Cell suspensions and cell-free extracts:

The washed cells were suspended in 0.1 M phosphate buffer of pH 7.8 containing 0.01 % 2-mercaptoethanol in the proportion of 2 mg cells as dry matter per ml of the solution and subjected to a Ratheon 10 kc sonic oscillator for 20 min. The intact cells and debris were removed by centrifugation at 11,000 r.p.m. for 25 min. The supernatant was dialyzed against 0.005 M phosphate buffer of pH 7.8 for 12 hr at 4 °C. The resulting solution was used as enzyme source.

Chemicals:

o-Aminobenzaldehyde was synthesized from o-nitrobenzaldehyde according to the procedure of Smith and Opie (85). D-Lys, D-Phe and D-Val were kindly provided by Dr. I. Chibata, Tanabe Pharm. Co. Ltd.

Analytical methods:

Amino acids were determined according to the method described previously (35, 36). Amino acids were separated by circular paper chromatography, using phenol saturated with water — ammonia (200:1), n-butanol — acetic acid — water (4:1:1) or ethanol — ammonia — water (18:1:1) systems. When a phenol solvent system was adopted, after development, the paper was washed with acetone and dried enough to remove remaining phenol. KGA was determined by modified methods of Carvallini et al. (86) and Friedemann et al. (87), after 2,4-dinitrophenylhydrazones were separated by paper chromatography. Protein was determined by the modified method of Lowry et al. (41).

Measurement of transaminase activity:

The activity of transaminase was routinely assayed by measuring the amount of the amino acid formed and a residual amino donor. The standard assay system consisted of 40 μmoles of amino acid, 40 μmoles of keto acid, 40 μg of pyridoxal phosphate, 100 μmoles of phosphate buffer of pH 8.0 and the enzyme in a final volume of 2.0 ml.

Incubation was carried out for 60 min. at 37°C, and the reaction was arrested by the addition of mixture of 0.9 ml of ethanol and 0.1 ml of 50 % trichloroacetic acid solution. After protein was removed, 10 ~ 50 µml aliquots of the supernatant were placed on circular paper chromatograph for analysis of amino acid. Appropriate blanks and controls which included sets for endogenous reaction were always run.

Activity was expressed as µmoles of amino acid formed per mg of protein per hr.

## Results

Transamination reaction by crude extracts:

The formation of Glu from KGA and several amino donors by the dialyzed crude extracts of five strains belonging to Achromobacteraceae, was shown in Table VII-1. Lys was transaminated with KGA as a most active amino donor in the presence of cell-free extracts of Fl. fuscum, Fl. flavescens and Ach. liquidum, while such a reaction was not observed at all in the preparations of the other strains. The discussion on characteristic of the general distribution of transaminase activities in these strains has been reported in the previous chapter.

Table VII-1. Transaminase activities of crude extracts

| Amino donor | Activity* | | | | |
|---|---|---|---|---|---|
| | Flavobacterium fuscum | Fl.flavescens | Ach.liquidum | Ach.superficialis | Al.faecalis |
| γ-ABA | 0 | 0 | 0 | 1.8 | 0 |
| β-Ala | 0 | 0 | 0 | 0.6 | 0 |
| L-Asp | 1.3 | 1.5 | 1.3 | 1.6 | 1.9 |
| L-Leu | 2.3 | 1.2 | 1.0 | 0 | 2.2 |
| L-Lys | 3.8 | 2.6 | 3.0 | 0 | 0 |
| L-Phe | 3.7 | 2.2 | 2.8 | 0 | 1.9 |

*: Glutamate formed (µmoles) per mg of protein per hr.

Reaction system: amino acid 40 µmoles, KGA 40 µmoles, PALP 40 γ, enzyme, pH. 8.0 phosphate buffer 100 µmoles in 2.0 ml. Incubation: for 1 hr. at 37°C.

Purification of transaminase:

The cell-free extract of Fl. fuscum dialyzed against 0.005 M phosphate buffer of pH 7.8 for 12 hr at 4°C was stored at -20°C for 15 hr. After thawing of the frozen solution, the precipitated residue was removed by centrifugation. The precipitate had no activity of transaminase.

To the supernatant, 1.5 % solution of protamine sulfate of pH 7.0, was added under stirring in proportion of 1 ml per 100 mg protein in enzyme solution. After 10 min, the resulting precipitate was removed by centrifugation.

The supernatant obtained was brought to 50 % saturation with ammonium sulfate. After 30 min, the precipitate was removed by centrifugation and the resulting supernatant was brought to 70 % saturation with ammonium sulfate. The pH of the solution was kept at 7.2～7.8 during the treatment. The precipitate was collected and dissolved in 0.01 M phosphate buffer of pH 7.8. One M acetate buffer solution of pH 7.3 (0.1 volume) was added to the solution and then the same amount of alumina gel Cγ as protein was suspended. After the mixture was kept under stirring for 10 min at 2°C, the gel was collected. The enzyme was eluted twice from the gel with 0.1 M phosphate buffer of pH 8.0, and both eluates were combined. Ammonium sulfate was added to the eluate to attain 50 % saturation and the precipitate formed was centrifuged. The supernatant was brought to 65 % saturation with ammonium sulfate. The precipitate was dissolved in 0.01 M phosphate buffer of pH 7.8.

Table VII-2. Partial purification of L-Lys — KGA transaminase

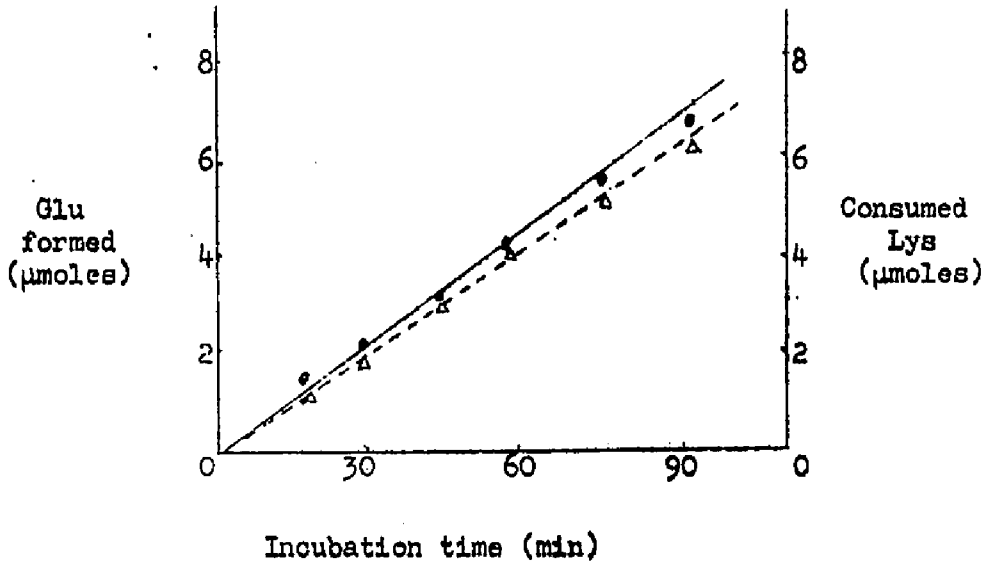
| Fr. No. | Enzyme fraction | Specific activity | Yield of activity | Activity ratio* Asp/Lys | Leu/Lys | Phe/Lys |
|---|---|---|---|---|---|---|
| 1 | Sonicate | 1.8 | 100 % | 0.31 | 0.61 | 0.95 |
| 2 | Freezing treatment at pH 7.8 | 4.7 | 96 | 0.28 | 0.62 | 0.98 |
| 3 | Protamine treatment | 6.1 | 78 | 0.50 | 0.60 | 0.58 |
| 4 | Ammonium sulfate fractionation I | 16.1 | 48 | 0.38 | 0.52 | 0.43 |
| 5 | Alumina gel Cγ treatment | 34.5 | 39 | 0 | 0.54 | 0.50 |
| 6 | Ammonium sulfate fractionation II | 41.8 | 27 | 0 | 0.37 | 0.44 |

* : Activities for reaction between KGA and Asp, Leu, Phe and Lys, respectively.

The enzyme was purified to about 2 8 fold with the overall yield of about 25～30 % by the above procedure (Table VII-2).

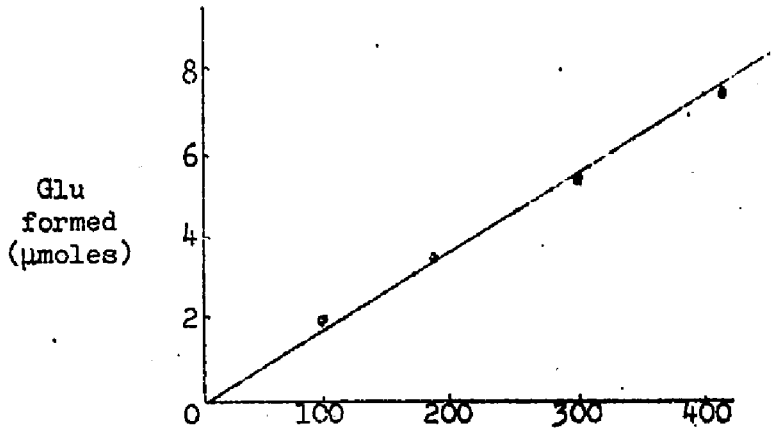
The relations between the transaminase activity, and protein concentration and incubation time are shown in Figs. VII-1 and VII-2.

Fig. VII-1. Effect of incubation period on the transaminase activity




—•—•— Formation of Glu. ---△---△--- consumption of Lys
Enzyme; 250 μg of fraction 4.

Fig. VII-2. Effect of enzyme concentration on the transaminase activity.




Enzyme; fraction 4.

Stoichiometry of reaction:

Table VII-3 shows that the formation of Glu proceeds in parallel with the disappearance of Lys and KGA in a complete system.

In the control system lacking KGA or Lys, Glu was not formed nearly at all and neither the amount of amino donor nor that of acceptor was changed during the reaction under this condition.

In the supernatant of the reaction mixture, no other amino acid and ketonic acid than Lys, Glu and KGA was detected by paper chromatography.

Table VII-3. Transaminase reaction between Lys and KGA

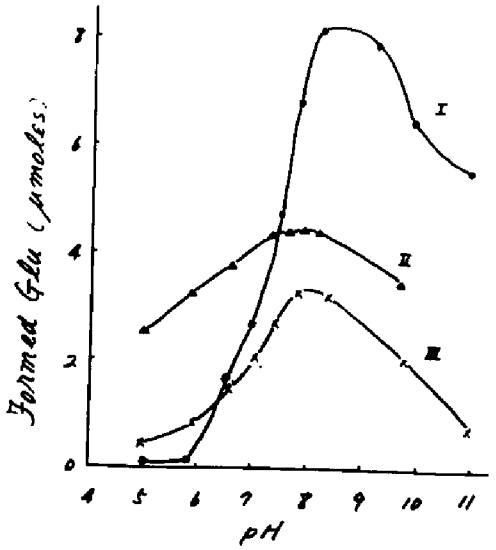
| Incubation period / Substrates or product | Substrates or product (μmoles) 0 min | 60 min | 120 min |
|---|---|---|---|
| L-Lys | 20 | 16.2 | 12.7 |
| KGA | 20 | 15.3 | 11.3 |
| Glu formed | 0 | 4.0 | 7.8 |
| L-Lys | 20 | 19.7 | 19.7 |
| KGA | 0 | 0 | 0 |
| Glu formed | 0 | 0 | 0 |
| L-Lys | 0 | 0 | 0 |
| KGA | 20 | 19.4 | 19.3 |
| Glu formed | 0 | 0.1 | 0.2 |

Incubation mixture: substrates, PALP (20 μg), enzyme (dialyzed Fr. 2), 1.0 mg and 50 μmoles of phosphate buffer of pH 8.0 in 1.0 ml. Incubation temperature; 37°C.

Effect of pH on activity:

With the standard reaction system, but with different buffers, the effect of pH on the activity of the transaminase was determined. The optimal pH of Lys-KGA trancaminase was found at about 8.3

Fig. VII-3. Effect of pH on the transaminase activity.




I. Lys — KGA
II. Asp — KGA
III. Leu — KGA

(Fig. VII-3). The activity fell sharply on the acid side of the optimal pH, but decreased a small amount on the alkaline side.

In the Asp — KGA and Leu — KGA systems, the optimal pH was between 7.2 and 8.0 and at about 7.8, respectively.

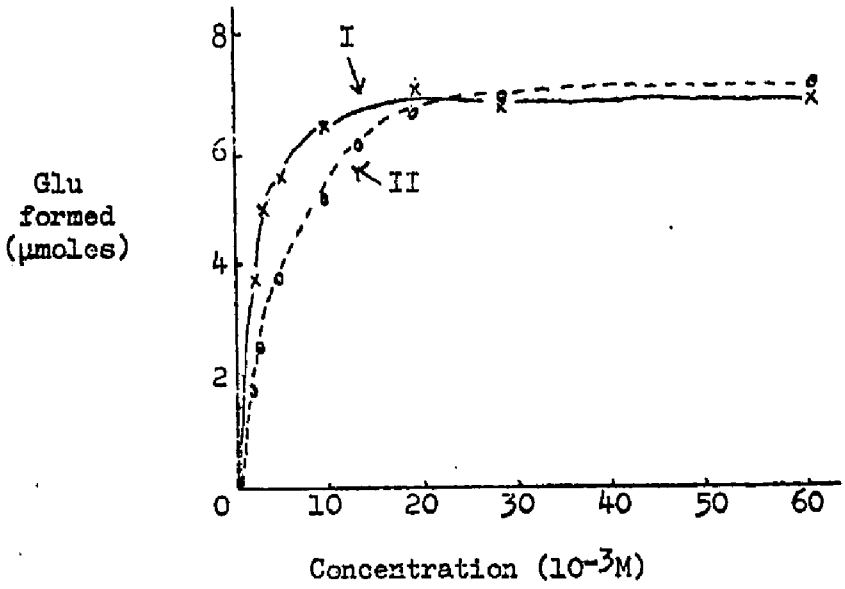
Effect of incubation temperature:

The rate of transamination increased with incubation temperature in a linear relationship between 20° and 37°C. The maximal activity was found to be at about 45 °C and decreased sharply over this point in the condition employed.

Effect of substrate concentration:

The transaminase activities with different concentration of Lys and KGA are shown in Fig. VII-4. From these results the Km values for Lys and KGA, as calculated from the Linewerver-Burk plot, were approximately $4.3 \times 10^{-3}$M and $2.3 \times 10^{-3}$M, respectively.

**Fig. VII-4. Effect of concentration of substrate on the transaminase activity**




I. Concentration of KGA changed at $4 \times 10^{-2}$M of L-Lys.

II. Concentration of L-Lys changed at $4 \times 10^{-2}$M of KGA.

Effect of inhibitors:

Table VII-34 shows the effect of inhibitors on the transaminase activity. The activity was increased by the addition of small amount of PALP, but the enzyme preparation employed in this experiment showed the reasonable activity without the addition of PALP to the reaction mixture. Penicillamine, known as an antivitamin-$B_6$ agent,

Table VII-4. Effect of inhibitors on the transaminase activity

| Inhibitor | Concentration (M) | PALP(γ/ml) | Activity |
|---|---|---|---|
| None | | 20 | 6.7 |
| None | | 0 | 4.8 |
| DL-Penicillamine | $2 \times 10^{-2}$ | 0 | 1.4 |
| " | $2 \times 10^{-3}$ | 0 | 3.6 |
| " | $2 \times 10^{-4}$ | 0 | 4.2 |
| " | $2 \times 10^{-5}$ | 20 | 4.7 |
| Hydroxylamine | $2 \times 10^{-4}$ | 0 | 2.8 |
| Thiosemicarbazide | $10^{-3}$ | 0 | 3.6 |
| Semicarbazide | $10^{-3}$ | 0 | 2.7 |
| KCN | $10^{-3}$ | 0 | 4.6 |

showed the fairly inhibitory action on the transaminase activity, especially at relatively high concentration. The reactivation of transaminase inhibited by DL-penicillamine was achieved by the addition of PALP. Hydroxylamine, thiosemicarbazide and semicarbazide inhibited the activity, but it is not suggested whether these carbonyl reagents inhibited the enzyme activity by combining with PALP , or whether they showed inhibition by reacting with an amino acceptor. KCN had no effect.

**Optical specificity:**

The partially purified preparation also catalyzed transamination between KGA and various other amino acids than Lys, while it did not show the activity of reaction of Lys with PyA, OAA or PPA.

Table VII-5. Optical specificity of the transaminase

| Amino donor | Activity |
|---|---|
| None | 0.56 |
| L-Lys | 5.80 |
| D-Lys | 0.75 |
| L-Phe | 5.40 |
| D-Phe | 0.68 |
| L-Val | 3.70 |
| D-Val | 0.65 |

Table VII-5 shows that only L-Lys was transaminated with KGA and the D-enantiomorph was not utilized as an amino donor in Lys — KGA transaminase system. Glu was formed from neither D-Val nor D-Phe as an amino donor, while transamination between L-Phe or L-Val and KGA was remarkably observed in the preparation.

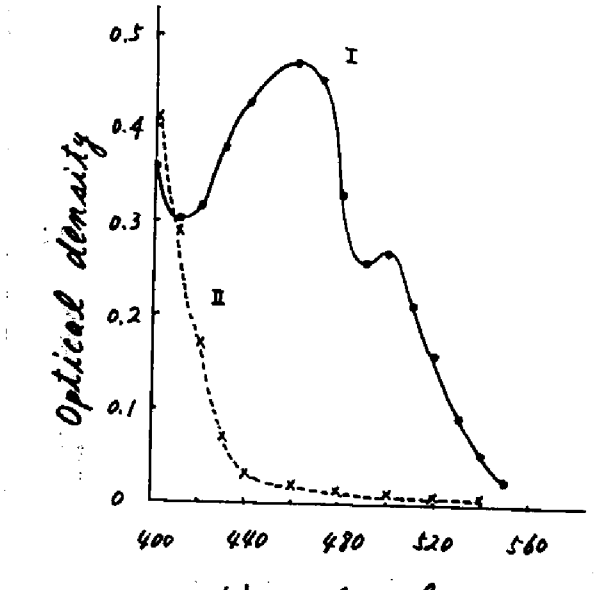
The nature of the keto analogue of L-Lys transamination:

One ml aliquot of the supernatant of reaction mixture was treated with 2 ml of 2,4-dinitrophenylhydrazine solution (0.4 % in 2 N HCl) for 30 min at 37°C, and the resulting hydrazones were extracted with 10 ml of ethyl acetate. The ethyl acetate layer was washed twice with water and then extracted with 0.5 ml of 10 % sodium carbonate solution. An aliquot of aquous layer was subjected to paper chromatography. Any other new spot than hydrazone of KCA was not detected on paper with two different solvent systems; n-butanol-ethanol-water (5:1:4) (upper layer) and isopropanol-water-ammonia (120:20:10).

Reaction with o-aminobenzaldehyde:

When portions of L-Lys transaminase reaction mixture were heated with o-aminobenzaldehyde solution, an orange-yellow color was developed. The color was fully developed by heating for 15 min in a boiling-water bath, and was only a little affected by the change of pH values between 4.5 and 6.5, although it was faintly decreased at pH over 7.0.

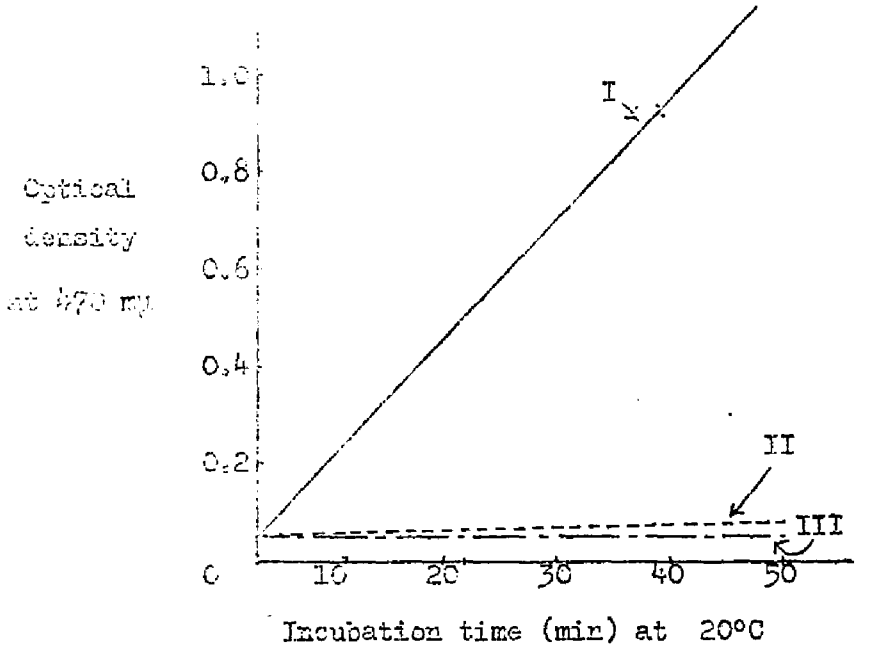
Fig. VII-5. Adsorption spectra of colored solution with o-aminobenzaldehyde.




I. Complete system
II. minus Lys

Fig. VII-5 shows that the adsorption spectra of the resulting color has a peak between 450 and 470 mμ. It was found that the enzyme reaction could be carried out in the presence of the reagent and the color was developed stably under the incubation condition employed as reported in the study of Orn transaminase reaction by Pincham (22). The development of color could be pursued in a

Fig. VII-6. Color reaction with o-aminobenzaldehyde.




I. Complete system : L-Lys 20 μmoles, KGA 20 μmoles, PALP 10 μG, o-aminobenzaldehyde 10 μmoles, enzyme and pH 8.0, phosphate buffer 100 μmoles in 5.0 ml.
II. D-Lys was used in the place of L-Lys.
III. Control system lacking KGA or L-Lys, or used boiled enzyem.

photoelectric colorimeter (Fig. VII-6). The color development progressed almost in parallel with the formation of Glu and disappearance of Lys.

Control reaction system lacking L-Lys or KGA did not reveal any coloration and no coloration was observed by the reaction mixtures with reactants when the boiled enzyme was employed. L-Lys could not be replaced by its D-isomer.

Reaction with p- dimethylaminobenzaldehyde:

Two ml aliquots of the supernatant of reaction mixture were heated with 2 ml of 0.1 M phosphate buffer solution of pH 6.5 and 1 ml of 2 % p-dimethylaminobenzaldehyde solution in ethyl cellosolve in a boiling-water bath for 20 min, according to Simura and Sagisaka's method (88). The intensity of the developed orange yellow color was read at 470 mμ.

Table VII-6 shows that this reagent gave a strong color with the supernatant of complete system, but no color with that of control systems lacking L-Lys or KGA, or employing boiled enzyme.

Table VII-6. Color reaction with p-dimethylaminobenzaldehyde

| Reaction system | Optical density at 470 mμ |
|---|---|
| Complete system* | 1.490 |
| minus L-Lys | 0.050 |
| minus KGA | 0.027 |
| minus enzyme | 0.024 |
| Boiled enzyme used | 0.030 |
| D-Lys used | 0.128 |

*: complete system; L-Lys 40 μmoles, KGA 40 μmoles, PALP 40 μg, pH 8.0 phosphate buffer 100 μ moles, in 2.0 ml. Incubation was carried out at 37°C for 30 min.

The system containing D-Lys in place of its L-isomer was only faintly positive to the test.

## Discussion

It has been believed that Lys can be very little or not at all transaminated in the living system. Meadow and Work (90) reported briefly that acetone-dried cells of such bacteria as E. coli mutant, Bacillus cereus and Sarcina lutea, catalyzed some transamination between L-Lys or D-Lys and PyA, OAA or KGA.

The enzyme preparation of Fl. fuscum described in the present chapter could catalyzed the transamination reaction between L-Lys and KGA to form Glu and its activity was very high in comparison with those of the other transaminases. The transaminase was partially purified, although the preparation obtained showed some transaminase activity of amino acids other than L-Lys. The formation of Glu progressed stoichiometrically in parallel with the disappearance of L-Lys and KGA. D-Lys could not replace L-Lys as an amino donor and neither PyA, OAA nor PPA was used as an amino acceptor to L-Lys in this system.

Recently, Ichihara and Suda (46) found that δ-aminovaleric acid was formed oxidatively with carbon dioxide and ammonia from L-Lys, and transaminated with KGA to produce L-Glu and glutaric semialdehyde by the specific transaminase in a strain of Pseudomonas.

While in this experiment, only Lys and Glu were detected by ninhydrin reaction on paper in the supernatant of the reaction mixture after incubation for 60 min, and no other amino acid than Lys was observed in the control system lacking KGA. In the experiment

by the resting cells with a control system without adding an amino acceptor, the formation of δ-aminovaleric acid was not observed.

It has been reported that glutaric semialdehyde, the transaminated product of δ-aminovaleric acid, reacts with 2,4-dinitrophenylhydrazine to form its hydrazone.

However, when the supernatant of the reaction mixture was reacted with 2,4-dinitrophenylhydrazine, only hydrazone of KGA was located on paperchromatograph with two different solvents which hydrazones of glutaric semialdehyde and KGA were easily separated with.

It is obvious that the reaction observed here is not attributed to the oxidation of Lys to δ-aminovaleric acid and the following transamination between the latter and KGA, but Glu was formed directly by the enzymatic transamination of L-Lys with KGA.

The L-Lys transaminase activity was inhibited by the addition of penicillamine at a relatively high concentration and was somewhat recovered by the addition of PALP, the activity was increased by about 30 % by the extraneous supply of the cofactor.

Resolution of bacterial transaminase to apoenzyme and PALP varied with the kinds of the enzymes, e.g. γ-ABA — KGA transaminase of Ach. superficialis could reveal full activity without the addition of cofactor throughout the purification process, while Asp — KGA transaminase released easily the coenzyme during the process and the activity was recovered by the addition of PALP as reported previously by author (35,36). From this point of view, the L-Lys transaminase was suggested to be an intermediate type between these two transaminases mentioned above.

The Lys transaminase system was unique among the other transaminase systems in yielding no new ketonic acid detectable as its 2,4-dinitrophenylhydrazone on a chromatograph after an experiment in which enzyme preparation and substrates were incubated in phosphate buffer.

o-Aminobenzaldehyde was reacted with the supernatant of the incubated reaction mixture by heating to form an orange yellow color. This color reaction, which might probably arise from formation of the dihydroquinazolinium compound (90), advances the suggestion that the keto analogue of L-Lys be present as a cyclic compound as reported on Orn transamination (22). Furthermore, p-dimethylaminobenzaldehyde

gave a dark yellow color with the supernatant, while Shimura and Sagisaka (15) has reported that $\Delta^1$-piperidine-2-carboxylic acid which was regarded as a biosynthetic intermediate from α-aminoadipic acid to Lys in yeast, reacted with this reagent to form the similar color.

These three facts: (1) A new ketonic acid hydrazone was not detected, (2) o-Aminobenzaldehyde gave the unique color with the supernatant of reaction mixture. (3) The color test with p-dimethylaminobenzaldehyde was positive, suggest that the carbonyl group initially formed may condense with the remaining amino group to form a piperidine ring, i.e. the keto analogue of L-Lys may be in the form of $\Delta^1$-piperidine-2-carboxylic acid or $\Delta^1$-piperidine-6-carboxylic acid under the condition of the experiments. The open form might be in equilibrium with the ring form and the equilibrium position might be enough towards the latter and away from the former. The position of amino group of L-Lys transaminated has not been elucidated and further work will be continued.

## Summary


1. Transaminase reaction between L-Lys and KGA was found in the cell-free extracts of Fl. fuscum, Fl. flavescens and Ach. liquidum.
2. The transaminase in the extract of Fl. fuscum was partially purified and some properties were investigated.
3. The formation of Glu proceeded stoichiometrically with the disappearance of the substrates by transaminase action.
4. D-Lys and PyA, PPA or OAA could not participate in this reaction as an amino donor and an amino acceptor, respectively.
5. The activity of the L-Lys transaminase was inhibited by the addition of penicillamine and stimulated by the addition of PALP.
6. As the keto analogue of L-Lys did not react with 2,4-dinitrophenylhydrazine to form its hydrazone, but reacted with o-aminobenzaldehyde and p-dimethylaminobenzaldehyde to produce each unique color, it was suggested to exist in a form of a cyclic compound containing a piperidine ring.

VIII. General Discussion.

The quantitative determination procedure of amino acid employed in the present study by means of circular paper chromatography and ninhydrin reaction in the presence of copper ion, was suitable for the assay of transaminase activity as a simple and accurate method.

The distribution of transaminase in the representative organisms of microorganisms employed in the present investigation was summarily listed in Table VIII-1.

With the exception of Clostridium acetobutylicum, all the strains of microorganisms revealed generally strong or very strong activity of Asp — KGA transaminase. In addition, KGA was most actively utilized as an amino acceptor among various keto acids. These facts suggest that Glu or KGA and Asp or OAA have the great physiological importance and Asp — KGA (or Glu — OAA) transamination may be regarded as the leading reaction in amino acid metabolism in microorganisms. The central role of Glu and Asp in these processes is shown in Fig. VIII-1. Glu is formed from KGA and inorganic ammonia by Glu dehydrogenase and Asp is produced from fumarate and inorganic ammonia by aspartase.

The fact that Ala metabolic function, i.e. the activity of L-Ala transaminase, D-Ala transaminase or Ala racemase, is very active as well as Asp transaminase in some bacteria belonging to Bacillus, suggests that Ala — KGA (or Glu — PyA) transaminase system may play an important role more than or as well as Asp — KGA reaction and be coupled with Ala-dehydrogenase system as demonstrated by Hong, Shen and Braunstein (91,92).

Leu —, Ileu —, Nval — and Val — KGA transaminase reactions were uniformly found in most strains and such analogous amino acids with side chain may be acted by the same or similar enzyme or enzymes. Although Leu — KGA transaminase reaction, a representative of this group, occurs in most microorganisms, some organisms lacking or with a faint activity of this enzyme reaction, e.g. Bacillus roseus, Ach. superficialis and Aspergillus oryzae, have active transaminations of α-Ala, β-Ala, Y-ABA or α-ABA. Reversibly, microorganisms having the active Leu transaminase system, shows no or little Ala transaminase, or Y-ABA or β-Ala transaminase activity. It is interesting that the occurrence of Leu transaminase group does not coincide with

that of Ala transaminase group, e.g. in B. roseus and Asp. oryzae, and with that of Y-ABA and β-Ala transaminase systems, e.g. in Ach. superficialis.

D-Ala transaminase reaction was found in some strains belonging to Bacillus and Pseudomonas, but not at all in some fungi or fungi imperfecti which have relatively active L-Ala transaminase system.

The occurrence of L-Lys transaminase reaction was demonstrated specifically in bacteria of Flavobacterium, Xanthomonas and Pseudomonas.

The wide distribution of Phe — KGA transaminase reaction suggests that Phe is also situated as an important metabolite.

The fractionation of bacterial transaminase and the investigation of enzyme properties come to the conclusion that several separate transaminases catalyze the various reactions and they require PALP as a cofactor and optimal pH presents in the weak alkaline side.

It is very interesting that such ω-amino acids as Y-ABA , β-Ala, taurine and Orn, are actively transaminated with KGA beyond expectation in Ach. superficialis and Ach. polymorph, although the physiological mechanism for such phenomena has not yet illustrated.

The first clear evidence for the existence of L-Lys — KGA transaminase reaction in the resting cells or the cell-free extract of Fl. fuscum, Fl. flavescens and Ach. liquidum was obtained and the properties of the enzyme were investigated. The L-Lys transaminase requires PALP as a prosthetic group, although the resolution was not completely attained. D-Lys are not utilized as an amino donor for KGA.

The results of study on keto analogue of L-Lys show that α- or ε-amino group of L-Lys is directly transaminated to KGA to form Glu, and the carbonyl group initially formed may condense with the remaining amino group to form a piperidine ring.

These observation will elucidate the unknown parts of biosynthetic pathway and degradation mechanism of L-Lys in the living system.

Table VIII-1. Summary of the distribution of transaminase reaction with KGA in microorganisms

(Relative activity of transaminase is represented as follows. 0: none or trace, 1: weak, 2: moderate, 3: strong, 4: very strong)

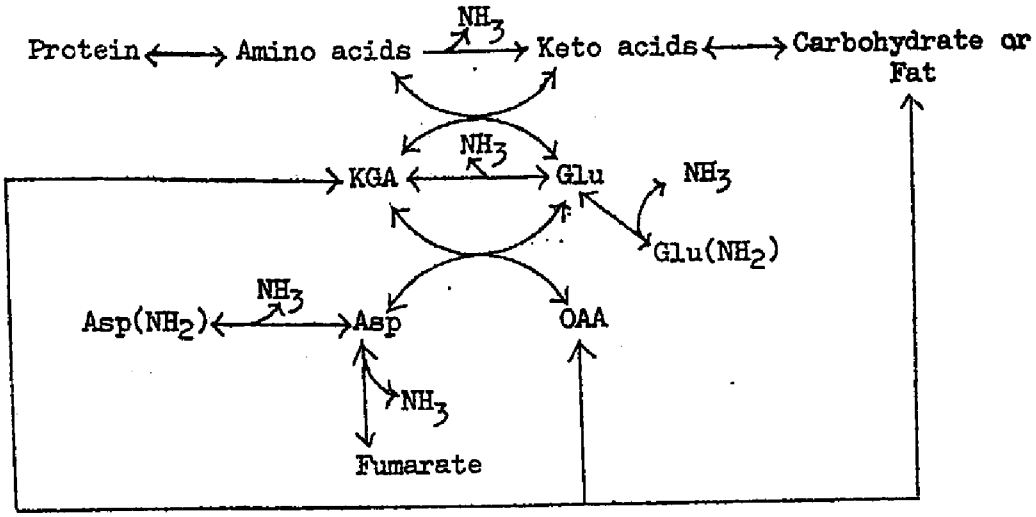
| Microorganism \ Amino donor | α-ABA | Y-ABA | L-Ala | D-Ala | β-Ala | Asp | Leu | Lys | Met | Orn | Phe | Try | Val |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Flavobacterium fuscum | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 4 | 3 | 2 | 4 | 0 | 3 |
| " flavescens | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 2 | 2 | 2 | 4 | 0 | 3 |
| Achromobacter superficialis | 0 | 3 | 0 | 0 | 2 | 3 | 0 | 0 | 0 | 2 | 1 | 1 | 0 |
| " polymorph | 0 | 3 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 |
| " liquidum | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 3 | 3 | 2 | 3 | 2 | 1 |
| Escherichia coli 2b | 0 | 0 | 0 | 0 | 0 | 3 | 2 | 0 | 1 | / | 3 | 1 | 2 |
| Alcaligenes faecalis | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 1 | / | 2 | 1 | 2 |
| Agrobacterium tumefaciens | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| Xanthomonas citri | 1 | 0 | 0 | 0 | 0 | 4 | 4 | 2 | 2 | 3 | 4 | 3 | 4 |
| Corynebacterium sepedonicum | 1 | 1 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 3 | 1 | 0 | 1 |
| Proteus vulgaris | 2 | 0 | 3 | 0 | 0 | 4 | 1 | 0 | 2 | 0 | 4 | 3 | 1 |
| Pseudomonas fluorescens | 2 | 4 | 3 | 0 | 0 | 4 | 4 | 2 | 3 | 1 | 3 | 3 | 3 |
| Bacillus natto SN | 4 | 2 | 4 | 4 | 0 | 4 | 1 | 0 | 1 | 3 | 1 | 1 | 1 |
| " roseus | 4 | 0 | 4 | 4 | 0 | 3 | 0 | 0 | 1 | 4 | 0 | 1 | 1 |
| " thiaminolyticus | 3 | 0 | 2 | 0 | 2 | 3 | 4 | 0 | 4 | 1 | 2 | 1 | 4 |
| Clostridium acetobutylicum | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 2 | / | 1 | / | 3 |
| Aspergillus oryzae | 1 | 0 | 4 | 0 | 0 | 3 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| " tamarii | 1 | 0 | 1 | 0 | 0 | 4 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |

(continued)

| Microorganism \ Amino donor | α-ABA | Y-ABA | L-Ala | D-Ala | β-Ala | Asp | Leu | Lys | Met | Orn | Phe | Try | Val |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Penicillium chrysogenum | 1 | 0 | 2 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| " expansum | 0 | 0 | 4 | 0 | 0 | 4 | 3 | 0 | 0 | 2 | 4 | 3 | 3 |
| Rhizopus batatus | 0 | 1 | 1 | 0 | 0 | 4 | 1 | 0 | 0 | 3 | 1 | 0 | 1 |
| Mucor rouxii | 0 | 2 | 0 | 0 | 0 | 3 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| " circinelloides | 0 | 2 | 0 | 0 | 0 | 3 | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| Monascus anka | 1 | 1 | 1 | 0 | 1 | 3 | 1 | 0 | 1 | 0 | 2 | 1 | 1 |
| Neurospora crassa | 1 | 1 | 1 | 0 | 0 | 3 | 2 | 0 | 1 | 1 | 1 | 0 | 1 |
| " sitophila | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 2 | 0 | 1 | 0 | 0 |
| Gibberella fujikuroi | 0 | 0 | 3 | 0 | 0 | 2 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| Fusarium lini | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Pullularia pullulans | 1 | 0 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| Streptomyces ruber | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 2 | 2 | 0 |
| Actinomyces asteroides | 2 | 0 | 0 | 0 | 0 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 1 |
| Candida rubra α | 0 | 2 | 0 | 0 | 0 | 4 | 4 | 0 | 3 | 3 | 4 | 4 | 4 |
| Saccharomyces sake No. 7 | 0 | 0 | 0 | 0 | 0 | 4 | 4 | 0 | 3 | 0 | 3 | 2 | 3 |
| Debaryomyces japonicus | 0 | 2 | 0 | 0 | 0 | 4 | 4 | 0 | 4 | 0 | 4 | 4 | 2 |
| Zygosaccharomyces major | 2 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 1 | 0 | 1 | 0 | 2 |
| Endomyces lindneri | 0 | 0 | 0 | 0 | 0 | 2 | 4 | 0 | 4 | 0 | 4 | 4 | 2 |

(Methods and conditions of experiments; see the text.)

Fig. VIII-1. Some important reactions in the synthesis and degradation of amino acids in microorganisms.



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